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MicroRNA regulation of prostate cancer desensitization to androgen receptor antagonist drugs during androgen deprivation therapy

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MICRORNA REGULATION OF PROSTATE CANCER DESENSITIZATION TO ANDROGEN RECEPTOR ANTAGONIST DRUGS DURING ANDROGEN DEPRIVATION THERAPY

by

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ABSTRACT

The current standard treatment of prostate cancer by androgen deprivation therapy involves using drugs such as bicalutamide (Casodex) to antagonistically block androgen receptors that are normally present within prostate cells. Usually, the therapy is successful in the short run at limiting the growth of prostate cancer. However, in virtually all cases tumors begin to grow aggressively again after several months of treatment and new therapies must be started. The mechanism by which these prostate cells transform from androgen sensitive to androgen independent and anti-androgen resistant is unclear. In this study, we investigated the role of microRNAs, small 15 to 18 nucleotide regulatory RNAs, in regulating the desensitization of prostate cancer cells to the androgen receptor antagonist drug bicalutamide.

In order to identify significant microRNAs, quantitative PCR was used to obtain genome-wide microRNA expression levels of 885 human microRNAs at different timepoints for androgen sensitive LNCaP cancer cells treated with bicalutamide and for untreated control cells in tissue culture. Analysis of microRNA expression by clustering analysis and by statistical comparisons of treatment groups resulted in identification of 28 microRNAs that have altered expression in the progression process. *In silico* target prediction analysis was performed with the microRNAs shown to have altered expression, and a group of genes predicted to be under microRNA regulatory control during cancer progression to resistance was identified. A microRNA expression profile can be useful in developing more effective prognostic and therapeutic tools for prostate cancer.

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CONTRIBUTIONS

Richard Ottman

- LNCaP cell culture and Casodex treatment for microRNA screening experiment
- RNA isolation and reverse transcription
- Quantitative PCR

Robert Lorch

- LNCaP cell culture and Casodex/DHT treatment for western blot experiment
- Assistance with quantitative PCR
- MicroRNA expression data analysis
- Target prediction analysis

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INTRODUCTION

Prostate cancer in men represents one of the most persistent challenges to modern medicine both in terms of its widespread rate of occurrence and in its evasiveness to treatment. The statistics for prostate cancer are astonishing; according to American Cancer Society estimates, 1 in 6 men will be diagnosed with prostate cancer in their lifetime and 1 in 36 will die from it. The Center for Disease Control and Prevention reports prostate cancer as the most diagnosed cancer in men for all races including populations of Hispanic origin with a combined incidence rate of 156.9 in 100,000. All fields of cancer research, and especially those dealing with prostate cancer, require our continued dedication in advancing our understanding of the mechanisms of carcinogenesis and progression as well as the optimal clinical treatments.

BACKGROUND

Clinical Treatment of Prostate Tumors

The majority of patients who present with well localized prostate carcinoma undergo radical prostatectomy, radiation therapy, or active surveillance. When prostate cancer is not well localized, standard therapy for patients with more metastatic but androgen-sensitive disease states is the administration of a combination of drugs used to simultaneously block androgen receptor function in prostate tissue as well as deplete the levels of androgen produced by the testes. The therapeutic basis of this tactic is that prostate cancer tissue requires stimulation by androgen in order to continue to survive and proliferate. Androgen depletion can be achieved by orchiectomy or by using a gonadotropin-releasing hormone (GnRH) analogue that decreases the production of luteinizing hormone in the pituitary by strongly binding the GnRH receptor and causing its down-regulation. At the same time, non-steroidal anti-androgen drugs such as flutamide (Eulexin), nilutamide (Nilandron, Anandron), or bicalutamide (Casodex) are used to antagonistically block prostate androgen receptors (Sharifi *et al.*, 2010). From this point on the disease course is fairly predictable. First, there is a noted decline in serum prostate specific antigen (PSA) level and a regression of the tumor. After a period of time, usually 18 to 24 months, PSA levels begin to rise, tumor progression returns, and prostate cancer symptoms become more pronounced (Chen *et al.*, 2009). This stage of prostate cancer is characterized by resistance to the anti-androgen and androgen deprivation therapy as well as more aggressive

metastatic phenotypes. Patients with metastatic androgen independent prostate cancer must seek stronger chemotherapies such as Docetaxel and Estramustine (Petrylak *et al.*, 2004).

It should be noted that the clinical use of anti-androgen drugs for the treatment of prostate cancer has potential widespread detrimental effects on the body. Some of the long-term effects of androgen deprivation therapy include fatigue, loss of bone density, decreased libido and sexual function, and increases in the rates of diabetes and cardiovascular disease (Alibhai *et al.*, 2006). Decrease in a patient's quality of life could also come as a result of a decrease in cognitive function, though this relationship is not well established (Alibhai *et al.*, 2010; Tadros and Garzotto, 2011). Nevertheless, it is apparent that the long-term side effects of androgen deprivation therapy and the certain progression to drug resistance warrants improved clinical screening techniques for patient-specific drug effectiveness as well as investigation into new therapies.

Androgen Receptor

The primary ligands for normally functioning androgen receptor are the lipophilic steroid hormone testosterone and the testosterone derivative dihydrotestosterone (the product of 5α-reductase). Androgen signaling is crucial for regulating the development and differentiation of the male reproductive organs, including the prostate, as well as the secondary sex tissues. Androgen and its receptor continue to mediate the normal maintenance and function of these tissues throughout life. Androgen receptor itself is present in the cytoplasm of prostate (and other) tissues in its inactive form as a 110 kDa class I steroid receptor protein bound to various

stabilizing proteins such as hsp90, hsp70, and hsp56 (Roy *et al.*, 2001). Lipophilic steroid androgens are able to diffuse across the lipid bilayer of the cytoplasmic membrane, and upon binding to the ligand-binding domain of the androgen receptor, cause a conformational change that leads to dissociation of heat-shock proteins, phosphorylation by kinases, translocation to the nucleus, and dimerization (Mangelsdorf *et al.*, 1995).

In the nucleus, androgen receptor binds to specific sequences in the genomic DNA referred to as androgen response elements (AREs). Transcriptional co-activators are recruited to induce transcription of genes under androgen receptor control, like PSA (Riegman *et al.*, 1991). PSA is commonly used in research labs and clinics as a reporter of androgen receptor activity, with high serum levels of PSA suggestive of prostate cancer. In prostate tumors the expression of androgen-regulated genes leads to cell survival and proliferation. For example, a recent study showed that the expression of TM4SF1 is under androgen control and that TM4SF1 overexpression increases prostate cancer cell migration (Allioli *et al.*, 2011). There are also some lines of evidence that suggest androgen receptor has additional roles in activating growth factor pathways by direct protein-protein interaction, such as through interaction of androgen receptor and the transcription factor AP-1 (Fronsdal *et al.*, 1998). Androgen receptor pathways are involved in many cellular processes, but it is clear that the main result of androgen receptor signaling in prostate cancer is the survival of cancerous prostate cells.

Androgen Independence

The earliest work on the effects of hormones on prostate cancer was performed by Huggins and Hodges when they did their Nobel Prize winning work on androgen dependence of prostate carcinoma in humans and suggested androgen ablation (depletion of circulating androgen levels) as a treatment for prostate cancer (Huggins, 1957). Several studies have shown that steroidal anti-androgen drugs, like cyproterone acetate, and non-steroidal anti-androgen drugs repress growth of prostate cancer cells *in vitro* (Bologna *et al.*, 1995; Kokontis *et al.*, 1998). However, clinical use of these drugs leads to castration resistant relapse in virtually all cases. Several studies have successfully established androgen independent cell-lines by prolonged passage in hormone-depleted media. Androgen dependent LNCaP-104S cells begin to die in androgen-depleted media and show a biphasic response to androgen, with low androgen levels inducing growth and high androgen levels repressing growth but inducing greater PSA production (Kokontis *et al.*, 1994; Lee *et al.*, 1995). On the other hand, androgen independent LNCaP-104R cells exhibit unrepressed growth in androgen-depleted media. Interestingly, the growth of LNCaP-104R cells is repressed by treatment with androgen levels that normally induce proliferation of LNCaP-104S cells (Kokontis *et al.*, 1998).

The molecular mechanisms of progression from androgen sensitive to androgen independent during treatment with androgen receptor antagonist drugs like Casodex remains largely unknown. Several studies over the years have shown that androgen-independent prostate cells exhibit increased androgen receptor expression and activity (Culig *et al.*, 1999; Kokontis *et*

al., 1998). This increase in androgen receptor activity suggests a functional role for androgen receptor in the maintenance of proliferation in androgen independent cancer. There are basically three theories proposed for the role of androgen receptor in the acquisition of androgen independence: activation of androgen receptor by secondary ligand, ligand independent activation of androgen receptor, or activation of an androgen receptor bypass mechanism.

Some evidence points towards the possibility that the androgen receptor becomes activated by certain non-steroidal growth factors and cytokines like IGF-1, KGF, EGF, and IL-6 in the androgen independent state (Jenster, 2000). One study showed that a point mutation in androgen receptor changes the effects of cyproterone acetate and the non-steroidal anti-androgen hydroxyflutamide so that the receptor is capable of recruiting the same co-regulators as if it were activated by androgen, essentially conferring agonist properties on the anti-androgen molecules (Berrevoets *et al.*, 2002). Studies have also identified androgen receptor mutations that greatly increase the affinity of the androgen receptor steroid binding domain to very low androgen concentrations (Thin *et al.*, 2003). The increased affinity to androgen may mean that the extremely low amount of androgen produced during implementation of an androgen deprivation strategy can preferentially bind the receptor. Additionally, strong amplification of the androgen receptor seen in some androgen independent prostate tumor samples, as well as the LNCaP-104R cell line, could increase the ability of the receptor to be activated by secondary ligand, possibly at a site separate from the androgen-binding domain (Ford *et al.*, 2003).

On the other hand, it may be that a ligand is not required for activation. It has been shown that the androgen receptor is capable of ligand-independent interaction with the transcriptional coactivator SRC1 in the nucleus, causing transcription at certain promoters in the absence of androgen (Powell *et al.*, 2004). It has also been proposed that an androgen receptor bypass mechanism might be important to progression, mediated through cytokines or other survival factors. One recent microarray investigation found that the proteins TWIST1, VAV3, and DKK3 could be important to a bypass pathway, resulting in downstream expression of survival factors (Marques *et al.*, 2010).

MicroRNA

The apparent elusiveness of a direct mechanism for the regulation of prostate cell progression to CDX-resistance has led many to suggest that a more complex regulatory network is at play. In fact, the progression mechanisms of many cancer types appear to be mediated by complex regulatory processes consisting of protein and RNA elements. Recent evidence suggests that the progression of multiple human cancers, including prostate cancer, is mediated by a growing class of small non-coding RNAs called microRNAs (miRNA). These short, ~22 nucleotide-long, single-stranded RNAs regulate human genes by binding to imperfectly complementary sequences on the 3' un-translated region (3' UTR) of target mRNA transcripts. Binding of the miRNA and its associated silencing complex to the mRNA transcript inhibits translational elongation, thus leading to post-transcriptional negative gene regulation (see *Mode of Regulation*). In some instances there is potential for great complexity in gene regulation by

miRNAs, as studies have shown that a single miRNA may target up to 200 different genes and that a single gene can be regulated by many miRNAs (Lim *et al.*, 2005). The first miRNA identified, lin-4, was found to be implicated in the larval development of *C. elegans* (Lee *et al.*, 1993). Lin-4 was also the first miRNA to have its regulatory function characterized when it was discovered that lin-4 targets the mRNA transcript of the protein-coding gene lin-14 and temporally mediates transition between larval stages L1 and L2 (Wightman *et al.*, 1993). Since then, several hundred human miRNAs have been identified and implicated in a diverse array of cellular processes including differentiation, cell proliferation, apoptosis, stress response, immunity, transcription, and tumorigenesis.

Biogenesis

Genes encoding miRNAs are found throughout the human genome with a large number of miRNA genes believed to be transcribed as polycistronic units utilizing their own promoters (Cai *et al.*, 2004). However, some miRNAs have been found encoded in the intronic regions of other known genes (Lagos-Quintana *et al.*, 2001). MiRNA genes are transcribed by RNA Polymerase II into long transcripts of several thousand bases that are then cleaved by the RNase III enzyme Drosha and its cofactor DGCR8 while in the nucleus. The resulting pri-miRNA is ~70 nucleotides long and forms an imperfect hairpin-loop structure. Active transport of the pri-miRNA out of the nucleus and into the cytoplasm is mediated by the nuclear membrane proteins Exportin-5 and RAN-GTP. Once in the cytoplasm the pre-miRNA transcript comes into contact with the enzyme Dicer and the RNA-binding protein TRBP which cleave the hairpin structure from the transcript, leaving an imperfectly complementary miRNA/miRNA* duplex consisting

of the guide strand mature miRNA sequence and the passenger strand miRNA. The guide strand of the miRNA/miRNA* duplex is preferentially incorporated into a multi-protein RNA-induced silencing complex (RISC) referred to as the miRISC, similar to the siRISC involved in RNAi silencing (Kim, 2005; Lee *et al.*, 2002).

Mode of Regulation

Regulation of translation by miRNAs has been seen across eukaryotic and bacterial domains, and even in archaea. The bulk of miRNA research has focused on eukaryotic gene regulation, and it appears that differences exist in the modes of miRNAs translational repression between plants and animals. Generally, miRNA gene regulation in plants involves near perfect sequence complementarity between guide strand miRNA and mRNA target, inducing mRNA degradation through the RNAi pathway. Additionally the miRISC of plants binds the target mRNA transcript at sites within the coding region of the mRNA (Hannon, 2002). A conserved Argonaute protein in the RISC (Ago2 in humans) mediates the RNA-cleaving "slicer" activity of the complex where perfect miRNA/mRNA base pairing occurs. The only mammalian miRNA known to lead to mRNA cleavage is miR-196 when it binds to Hoxb8 mRNA (Yekta *et al.*, 2004). Human miRNAs generally do not exhibit perfect sequence complementarity and do not induce mRNA degradation. As part of the miRISC, the guide strand sequence targets binding of the miRNA to semi-complementary sites on the 3' UTR of target mRNA sequences. The translation of these target genes is repressed due to the association of miRISC with the transcript (Esquela-Kerscher and Slack, 2006; Pillai *et al.*, 2005). Two models have been proposed for miRNA-induced translational repression, and both involve processing bodies (PBs) in the

cytoplasm. PBs are known to mediate normal mRNA degradation and turnover. In the first model, the miRISC bound at the mRNA 3' UTR interacts with the 5' m7Gppp cap. This interaction interferes with recruitment of the E1F4E translation factor that is responsible for directing the transcript to the ribosome. Translationally repressed mRNAs are then directed to PBs for degradation or possibly storage. In the second model initiation is not affected, but binding of the miRISC to the 3' UTR directs the mRNA to PBs where translation cannot occur (Pillai, 2005).

Roles for MicroRNAs in Cancer

The relatively recent emergence of high-throughput miRNA expression profiling techniques has begun to shed light on the various roles that miRNAs play in cancer. Microarray studies have shown that aberrant miRNA expression is correlated with several human cancers and that many miRNAs can be classified as oncogenes or tumor suppressor genes. The first miRNAs to have their expression associated with human cancer were miR-15a and miR-16-1. They have been shown to target the tumor-suppressor survival gene Bcl-2 and to be down-regulated in B-cell chronic lymphocytic leukemia (Cimmino *et al.*, 2005). Other examples of cancer-related miRNAs are miR-143 and miR-145, which are down-regulated in many cancer cell-lines (breast, prostate, cervical, and lymphoid cancer) as well as in colorectal tumors (Iorio *et al.*, 2005; Michael *et al.*, 2003). The conserved expression pattern across multiple cancer types indicates a generalized tumor suppression function of these miRNAs. The miRNA oncogene miR-21 has been found to be greatly overexpressed in glioblastoma tumors where it inhibits

apoptosis (Chan *et al.*, 2005; Ciafre *et al.*, 2005). Up-regulation of miR-21 expression has also been shown in breast cancer and prostate cancer where this miRNA may potentially have an oncogenic function (Iorio *et al.*, 2005). The first class of miRNAs shown to regulate an oncogene was the let-7 miRNA family, which mediates expression of the Ras oncogene (Johnson *et al.*, 2005). It has been found that among lung cancer patients let-7 miRNA is most down-regulated in those that exhibit poor post-operative survival and overall poor prognosis. Additionally, expression of let-7 in lung cancer tissue has been shown to inhibit proliferation (Takamizawa *et al.*, 2004).

MicroRNA Regulation in Prostate Cancer

With the overwhelming evidence that miRNAs have roles in various cancers, it is not surprising that there are recent indications that several miRNAs are implicated in the tumorigenesis and progression of prostate cancer (Catto *et al.*, 2011). MiR-221 and miR-222 have been shown to be up-regulated in androgen independent prostate cancer cell lines and to target the p27/kip1 tumor suppressor gene (Sun *et al.*, 2009). High throughput microarray analysis of miRNA expression in prostate cancer cell lines shows that miR-21 expression is induced by androgen-stimulated androgen receptor and, indeed, that miR-21 alone is sufficient for maintaining a castration-resistant phenotype in prostate tumors *in vivo* (Ribas *et al.*, 2009). Interestingly, it has also been shown that miR-21 targets PDCD4 and PTEN, two known anti-survival genes (Lu *et al.*, 2008; Papagiannakopoulos *et al.*, 2008). MiRNAs of the miR-17-92 family target the degradation of E2F transcription factor mRNA. In fact, it has also been shown

in prostate cancer cell lines that transcription factors of the E2F family are responsible for transcription of miR-17-92 family miRNAs. It is believed that a miRNA-mediated regulatory feedback loop is in place to control E2F expression here.

Androgen-induced AR has also been shown to mediate expression of miR-125b. This miRNA inhibits translation of Bak1 and induces androgen independent growth in transiently infected prostate cancer cells (Shi *et al.*, 2007). One study found that miR-146a is down-regulated in androgen-independent prostate cancer cells and proposed a possible mechanism of androgen independence by showing the negative regulation of the ROCK1 oncogene by miR-146a (Lin *et al.*, 2008). The miRNA miR-145 is down-regulated in many cancers including prostate cancer and has been shown to target the BNIP3 gene. Low expression levels of miR-145 and high BNIP3 expression levels in prostate tumors are associated with unfavorable patient outcomes (Chen *et al.*, 2010). Given the numerous examples of miRNAs implicated in prostate cancer, it is hypothesized that miRNAs play essential roles in the progression of prostate cancers to Casodex resistance.

OBJECTIVE OF THE STUDY

This study aimed to investigate the deregulation of miRNAs in human prostate cancer and the possible functional roles of deregulated miRNAs in the progression of prostate cancer cells toward anti-androgen resistance. Several major aspects of the study should be noted. Our primary focus was the measurement of miRNA levels at timepoints during anti-androgen treatment so that we could track time dependent miRNA changes that may have regulatory consequences. Due to the apparently complex nature of the androgen independence mechanism, a time dependent approach to screening miRNA expression during progression was central to the experimental design. Another important aspect of the study was the comparison of miRNA expression levels in anti-androgen treated cells to timepoint-paralleled androgen depleted cells so that we could isolate miRNAs that are deregulated due to the effects of anti-androgen binding to androgen receptor (and possibly additional mechanisms) separate from the effects of general androgen deprivation. Few studies have been so precise in their distinction between the changes that occur during castration resistance and anti-androgen resistance. The third focus of the study was to make use of the enormous amount of bioinformatics research that has been carried out to develop miRNA target prediction techniques. Several labs have created algorithms with the aim of predicting which mRNA transcripts specific miRNAs target. These algorithms that take several binding factors into account and the prediction results are made publically available online. A large portion of this study utilized *in silico* analysis techniques.

MATERIALS AND METHODS

Experimental Plan

The experimental plan was comprised of two phases:

- **M1** – Data acquisition, consisting of cell culture and treatment, western blot, and miRNome screening by qPCR
- **M2** – Data analysis, consisting of statistical analysis of expression data and *in silico* target prediction

M1. Cell culture and cell treatment

Principle

The LNCaP cell line is an androgen sensitive prostate cancer cell line widely used in androgen deprivation experiments. Originally isolated from a lymph node metastasis of a primary prostate tumor, LNCaP cells express androgen receptor containing a T^{868} to A^{868} mutation in the steroid binding domain. This mutation increases the affinity of the receptor protein for some anti-androgen molecules, including bicalutamide, and allows the signaling effects of anti-androgens to be more easily studied. (Veldscholte *et al.*, 1992; Veldscholte *et al.*, 1990). LNCaP-104S and LNCaP-104R1 are the sub-lines used in this study. LNCaP-104S cells are androgen sensitive and require androgen stimulation for growth. The line was established by selecting an LNCaP clone that demonstrated maximal proliferation with stimulation by 0.1 nM of synthetic androgen (Kokontis *et al.*, 1994). The LNCaP-104R1 line was created by prolonged

passage in androgen depleted media for several months (80 to 100 passages) and is characterized as having a moderately aggressive drug resistant phenotype. LNCaP-104R1 cells grow unrepressed in androgen-depleted media but are repressed by levels of androgen that induce maximal LNCaP-104S proliferation (Kokontis *et al.*, 1994). LNCaP-104R1 cells were utilized so that miRNA expression during anti-androgen treatment could be compared to expression in an established androgen independent line.

Experimental design

Cell culture of androgen sensitive LNCaP-104S cells was maintained before drug treatment in DMEM/10% FBS and 1 nM DHT with passage at 70% confluency. Culture of androgen independent LNCaP-104R1 cells was maintained in DMEM/10% FBS with passage at 70% confluency. All treatments of LNCaP-104S cells were initiated by 48 hours of androgen depletion in DMEM/10% charcoal-stripped FBS (CS-FBS) media. For protein experiments, LNCaP-104S cells were treated with either 10 nM DHT or 5 μM Casodex (CDX) and harvested at zero hour, 24 hours, 48 hours, and 72 hours timepoints. For qPCR miRNA screening, LNCaP-104S cells were treated with 5 μM Casodex in DMEM/10% CS-FBS media or only DMEM/10% CSFBS and harvested at one week and three weeks timepoints. LNCaP-104S and LNCaP-104R1 cells were also harvested at zero hour timepoint (before treatment with CDX).

M1. SDS-PAGE and western blot

Principle

The standard technique for resolving and identifying proteins from extracted cellular lysates is SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) followed by transfer to a protein-binding membrane, such as a PVDF (polyvinylidene fluoride) membrane, and western blot procedure using a primary antibody specific for the protein of interest. SDS-PAGE involves denaturing extracted proteins by boiling in the presence of sodium dodecyl sulfate and with the reducing agent β-mercaptoethanol. Besides general denaturation, β-mercaptoethanol reduces any tertiary disulfide linkages. Sodium dodecyl sulfate binds to and linearizes denatured polypeptides and imparts a uniform negative charge on all peptides. In this way, all protein subunits can be resolved by electrophoresis based only on size. Electrophoretic separation is performed in a thin polyacrylamide gel. Small pores in the bis-acrylamide lattice allow smaller polypeptides to migrate at a faster rate toward the anode when an electric current is applied. A standard protein ladder is run simultaneously so that the sizes of proteins can later be ascertained.

Transfer of the separated proteins is achieved by applying an electric current in a direction such that the negatively charged polypeptides migrate out of the gel and bind to an adjacent piece of protein-binding membrane. After staining the membrane with India ink to visualize the sample lanes, blocking with milk proteins ensures that all the protein-binding sites on the membrane are unavailable. Next, each lane is incubated with an appropriate concentration

of primary antibody specific for the protein of interest. After several rounds of rinsing to remove excess antibody and another round of blocking, the blot is incubated with an appropriate concentration of secondary antibody. The secondary antibody specifically binds to the constant regions of all immunoglobulins produced by a given species. The secondary antibody that is used is determined by the source of the primary antibody. The secondary antibody is also conjugated to a chemical that provides a means of visualization, commonly the enzyme horseradish peroxidase (HRP) which produces a fluorometric molecule in the presence of its substrate. When HRP-conjugated secondary is used, the protein of interest can easily be visualized by addition of the HRP substrate and developing on autoradiography film or with a western blot visualization system.

Experimental design

LNCaP-104S cells were maintained in CS-FBS for 48 hours and then treated with CDX or DHT. Cell samples were harvested by trypsinization at the following timepoints: zero hour, 24 hours, 48 hours, and 72 hours. Cells were lysed using the freeze-thaw method with Halt phosphatase inhibitor. Protein concentration was measured by Bradford assay and proteins were resolved by SDS-PAGE separation. After transfer to PVDF membrane, western blot for androgen receptor was performed with mouse monoclonal IgG1 anti-AR primary antibody (U.S. Biological, Swampscott, MA) (1:2,500 in TBS-T/5% milk) and HRP-conjugated goat polyclonal IgG anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA) (1:20,000 in TBS-T/5% milk). GAPDH was used for loading control. Western blots were

visualized by adding West Femto chemiluminescent substrate (ThermoFisher Scientific, Waltham, MA) to the secondary-probed membrane and then exposing and developing on autoradiography film.

M1. Reverse transcription

Principle

Due to the requirement of template DNA when carrying out qPCR reactions for cell expression studies, it is usually necessary to convert the RNA isolated from a sample of cells to complementary DNA sequences (cDNA) for amplification. The reverse transcriptase enzyme was discovered in retroviruses that use it to insert viral genetic code into the DNA genomes of infected cells (Rodgers *et al.*, 1995). In the lab, reverse transcriptase isolated from retroviral sources is added to RNA samples along with oligo-deoxythymidine primers, which bind to poly-adenosine tails of mRNA transcripts. Small RNAs are not expressed with poly-adenosine tails so this step necessitates first adding poly-adenosine to all RNAs in the sample using a poly-adenylate ligase. Once tails have been added and cDNA has been created from the RNA sequences in the sample, expression screening by qPCR can commence.

Experimental design

Cells were harvested by trypsinization and pellets were lysed with SBI Cells-to-Cts lysis buffer. SBI DNase I buffer was used to degrade DNA in the lysate. The SBI System Biosciences QuantiMir Small RNA Quantitation kit was used to tag all small RNAs in the lysate with poly-

adenylate tails, bind universal oligo-deoxythymidine primers, and convert total RNA to cDNA with reverse transcriptase.

M1. Quantitative polymerase chain reaction

Principle

The quantitative polymerase chain reaction technique is used to quantify the number of DNA copies of any specific sequence in a mixture of template DNA. It is a derivative of the more simple DNA amplification technique called the polymerase chain reaction. The standard polymerase chain reaction (PCR) is used to amplify the number of copies of a specific DNA sequence present in a reaction mixture. The required components of a PCR reaction mixture are as follows: forward and reverse primers for the DNA sequence of interest, DNA polymerase with a high temperature range (such as Taq polymerase), dNTPS, optimized cationic PCR buffer (usually containing potassium and magnesium ions), and the template DNA mixture. Using a thermocycler, the reaction mixture is subjected to successive rounds of temperature changes each consisting of a DNA denaturation phase of ~95°C, a primer annealing phase of ~60°C, and a transcript elongation phase of ~72°C. After approximately 30 cycles, the number of copies of a DNA sequence of interest will be have been amplified by several orders of magnitude. PCR amplification is extremely useful and has become central to almost all other DNA research techniques.

Quantitative real time PCR (qPCR) takes advantage of the exponential nature of PCR amplification and the quantitative aspects of spectrophotometry to quantify the initial amount of

any specific DNA sequence in a sample of template DNA. One method of doing this is to add SYBR green reporter dye to the PCR reaction mixture and then perform the PCR reaction on a specialized quantitative thermocycler. SYBR green dye binds to double stranded DNA as it is synthesized in the PCR reaction. Upon binding to double-stranded DNA the emission properties of SYBR green dye changes so that it emits green light (λ of ~522 nm) when excited by a laser, allowing the amplification of DNA to be measured in real time. For most data analysis methods, the qPCR thermocycler measures the green light emission of each well in a reaction plate and then calculates the exact PCR cycle at which the total green light emission from a well reaches a predefined threshold value (i.e. the total amount of amplified dsDNA in a well reaches a predefined value). To be valid, the threshold fluorescence must fall within the exponential phase of DNA amplification. This cycle number is referred to as the cycle threshold value (Ct). When used in conjunction with reverse transcription, quantitative polymerase chain reaction is a powerful method of quantifying RNA expression levels of genes. Isolated RNA from a sample must first be converted to cDNA by reverse transcriptase, and then qPCR can be used to measure levels of cDNA.

Experimental design

Genome-wide human miRNAs were screened by reverse transcription to cDNA and then SYBR green qPCR reaction on an ABI 7900 HT real time thermocycler. MiRNA-specific primers were provided in the SBI System Biosciences miRNome profiling kit encompassing 885 human miRNAs and three endogenously expressed small RNAs for plate controls (Human U6 snRNA, RNU43 snoRNA, and Hm/Ms/Rt U1 snRNA). Additionally, universal oligo-

deoxyadenosine reverse primers provided in the SBI System Biosciences kit were used in the qPCR reaction. A Ct value was obtained for each miRNA that amplified without error. All plate controls were amplified without error.

M2. Delta-delta-Ct analysis

Principle

Several factors are considered during experimental design and multiple strategies have been developed for analyzing qPCR expression data. For experiments that require the calculation of an absolute quantity of template DNA, accurate analysis necessitates the creation of a standard curve using standard reactions of known template quantity and then comparison to the test Ct value. On the other hand, most expression experiments do not require absolute quantification, but rather make use of relative expression quantification compared to some control group. If this is the case, the most common method of data analysis is the delta-delta-Ct method (ΔΔCt method) (Cikos *et al.*, 2007).

The ΔΔCt method requires no standard curve, is generally easier to perform than the standard curve method, and involves a simpler calculation than most other analysis methods (Livak and Schmittgen, 2001). In addition to having to include a control group in the experimental design, the ΔΔCt method calls for the inclusion of a set of reactions to amplify endogenously expressed control genes (housekeeping genes) called plate controls to normalize the expression data for plate-wide variations in amplification efficiency. For most mRNA

expression studies plate control genes such as GAPDH are used, while in miRNA expression studies endogenously expressed housekeeping small RNAs are used.

To accurately analyze expression data using the ΔΔCt method, it is helpful to determine gene-specific average amplification efficiency for each gene being measured. This can be done several ways, but the gene-specific efficiency is usually calculated by running qPCR reactions of serially diluted template DNA for each gene, plotting the log concentration vs. Ct, and then calculating the efficiency as ___ (Yuan *et al.*, 2008). The ΔΔCt method results in a fold amplification value that incorporates the test gene expression and the reference gene expression. If the gene-specific amplification efficiencies of the sample reaction and plate control reaction, *Pc*, (test group, *T*, and reference group, *R*) are close to equal, the various efficiencies will cancel out and the equation for fold amplification value can be simplified quite a bit. The formula for fold amplification value determination with the assumption of equal amplification efficiencies is part of the Excel software included in the SBI System Biosciences Small RNA Quantitation kit and is as follows:

$$\frac{}{}$$

The resulting value is a ratio of test expression to reference expression and can be further analyzed in many ways. It should be noted that as a ratio of expression it is sometimes beneficial to transform the fold amplification value to the negative reciprocal value for visualization purposes and some statistical uses. For example, a miRNA that shows half the expression in the test group as it does in the reference group will have a fold amplification value of 0.5. This value

can be transformed to its negative reciprocal, -2.0, showing that the miRNA was down-regulated two-fold.

Experimental design

All Ct values obtained from qPCR screening were analyzed using the ΔΔCt method. For the initial analysis, the treatment group LNCaP-104S zero hour was designated as the reference group and compared to all other treatment groups to calculate fold amplification values. In the initial analysis all fold amplification values deregulated above three-fold (up or down) were considered to be significant. All ΔΔCt calculations were performed in Microsoft Excel.

M2. Cluster analysis

Principle

Much insight into the gene networks that regulate cellular processes can be offered by clustering analysis methods that attempt to identify groups of genes with related functions based on expression data (Gitter *et al.*, 2010). The dynamic and complex nature of miRNA regulation makes clustering approaches to data analysis very useful. Hierarchical clustering can identify large-scale expression patterns of groups of miRNAs that have the same regulatory functions, and offers a way to visualize such groupings. Hierarchical clustering involves calculating distances between groups of genes (based on expression values) and then determining clustering solutions by iterative processing of all possible groupings. Two of the main considerations of clustering analysis are the method of measuring distance or similarity between groups and the type of hierarchical clustering algorithm that is used. The clustering software Gene Cluster 3.0

offers several options for determining distance or similarity (correlation, absolute correlation, Spearman rank correlation, Kendall's tau, Euclidean distance, and city-block distance) and for hierarchical clustering method (centroid linkage, single linkage, complete linkage, and average linkage)(de Hoon *et al.*, 2004).

Experimental design

Hierarchical clustering was performed on the expression data obtained from the initial ΔΔCt calculations using the LNCaP-104S zero hour control. Data was clustered using correlation measurement for similarity and complete linkage. Both the miRNAs and the treatment arrays were hierarchically clustered and reordered. The analysis was visualized as a heat map and hierarchical clustering dendrogram with the visualization program Java Treeview (Page, 1996).

M2. Additional comparisons

Principle

After the initial calculations of fold amplification values using LNCaP-104S zero hour as the control group, the comparison groupings were altered to study specific aspects of progression to drug resistance. Since the objective of the study involved identifying outliers in the expression set, it was necessary to calculate the average changes in expression and average deviation from the mean value. The measure of central tendency most suitable for this type of expression data is the geometric mean due to the multiplicative nature of the expression values and the decimal form of all down-regulated expression values. The formula for geometric mean is as follows:

and once the geometric mean has been calculated the geometric standard deviation can be calculated as

where A_i denotes an individual fold amplification value. Once the standard deviation has been determined for each comparison set it is possible to determine z-scores in order to identify the miRNAs that show expression changes in the significant regions of either tail of the expression distribution. The standard formula for z-score is

The standard z-score method was used for determining the z-score for all expression values greater than or equal to 1.0. Z-scores for negatively regulated miRNAs were calculated by negative reciprocal transformation of the fold amplification value and then addition of 2 before subtracting the mean value and dividing by the standard deviation:

Adding two insures that the distance of the negative reciprocal-transformed fold amplification value from the mean fold-expression value is accurate, since baseline expression change in our data was defined as 1.0 (meaning, a value of 1.0 denotes no change in expression). Additionally, also due to defining 1.0 as the baseline expression change, all geometric mean values determined to be less than 1.0 were negative reciprocal transformed for use in subsequent calculations. In order to avoid the assumption that miRNA expression is normally distributed, z-scores were only used as a means of selecting an arbitrary threshold and not as a means of assigning numerical significance.

Experimental design

The comparisons used are outlined in Table 1. The comparisons using LNCaP-104S one week and three weeks CDX treatment timepoints and the respective LNCaP-104S CS-FBS media treatment timepoints as reference samples show the relative miRNA expression changes due only to CDX treatment throughout the treatment regimen. The third comparison, relating LNCaP-104S cells treated with CDX for three weeks to a reference sample of LNCaP-104S cells treated with CDX for one week, is another way of studying progressive changes in miRNA expression during a CDX treatment regimen. This comparison better represents miRNA expression changes in the scenario of CDX treatment of androgen sensitive prostate cancer in concert with androgen deprivation therapy, since androgen deprivation dependent expression changes are not being adjusted for. Finally, the comparison of LNCaP-104S cells treated with CDX for three weeks to LNCaP-104R1 zero hour cells is another way to isolate only CDX-dependent changes in miRNA expression. However, differences between this comparison and

the CDX versus CS-FBS comparisons are expected due to widespread cellular differences between the androgen sensitive LNCaP-104S cells and established androgen resistant LNCaP-104R1 cells.

Table 1) Cell lines, treatments, and timepoints for test and reference samples of Comparisons A through D

	Test Sample			Reference Sample		
	Cell Line	**Treatment**	**Timepoint**	**Cell Line**	**Treatment**	**Timepoint**
A	LNCaP-104S	CSFBS/5μM CDX	One week	LNCaP-104S	CSFBS	One week
B	LNCaP-104S	CSFBS/5μM CDX	Three weeks	LNCaP-104S	CSFBS	Three week
C	LNCaP-104S	CSFBS/5μM CDX	Three weeks	LNCaP-104S	CSFBS/5μM CDX	One week
D	LNCaP-104S	CSFBS/5μM CDX	Three weeks	LNCaP-104R1	CSFBS	Zero hour

MiRNAs shown to be up- and down-regulated in the comparisons of Table 1 were subjected to cross-comparison analysis using Venn diagrams and the Venny tool available online (http://bioinfogp.cnb.csic.es/tools/venny/index.html). Calculations of standard deviation and z-score were performed by the previously described method. Expression fold-changes greater than two standard deviations from the mean expression fold-change (z-score less than -2 or greater than 2) were considered significant at this point in the data analysis.

M2. Target prediction

Principle

In recent years there has been an attempt to develop public databases of miRNA targets so that researchers may draw conclusions from the huge amount of data becoming available. Some bioinformatic studies have resulted in the development of algorithm-based methods to predict the gene targets of known mature miRNA sequences based on several factors. The miRDB database presents predicted miRNA-target matches and assigns each match a score from 50 to 100, representing the probability that the given mRNA is actually targeted. The target predictions are calculated using an algorithm designed to take into account seed conservation, other seed types, base composition, secondary structure, and location on the 3' UTR. The developers used a machine learning strategy called Support Vector Machines (SVM) to systematically search known miRNA target data to optimize the algorithm. The database is public and can be searched online (http://mirdb.org/miRDB) (Wang, 2008; Wang and El Naqa, 2008). Another publically available database of miRNA target predictions, TargetScan Human

release 5.1, can be searched as well. TargetScan Human assigns a context score to predicted targets based on site-type contribution, 3' pairing contribution, local AU contribution, and position contribution (http:// targetscan.org/) (Friedman *et al.*, 2009; Grimson *et al.*, 2007; Lewis *et al.*, 2005).

Experimental design

MiRNAs identified as significantly deregulated were inputs in target scan analysis using the internet-based target predictions software program miRDB (http://mirdb.org/miRDB). Target prediction results returned for up-regulated and down-regulated miRNAs were ranked first by the number of hits from the respective array of candidate miRNAs, and secondly by the arithmetic average of miRDB scores. Due to the large number of predicted target genes, only targets that received hits from multiple miRNAs and that received at least one hit with an miRDB score of 80 or greater (for down-regulated miRNAs) or 90 or greater (for up-regulated miRNAs) were included in the final lists of predicted targets. The target predication program TargetScan Human (http://targetscan.org) was used for validation of predictions.

RESULTS

To verify the expression of androgen receptor and the responsiveness of the LNCaP-104S cell line to androgen stimulation, western blotting for androgen receptor was performed with treated samples. Total protein extracted from LNCaP-104S cells treated with DHT and cells treated with Casodex at timepoints of zero hour, 24 hours, 48 hours, and 72 hours was subjected to SDS-PAGE and western blot, shown in Figure 1. LNCaP-104S cells treated with Casodex and treated with DHT did not show significant changes in androgen receptor expression from the zero hour. Androgen receptor expression in the LNCaP-104S was verified by western blotting.

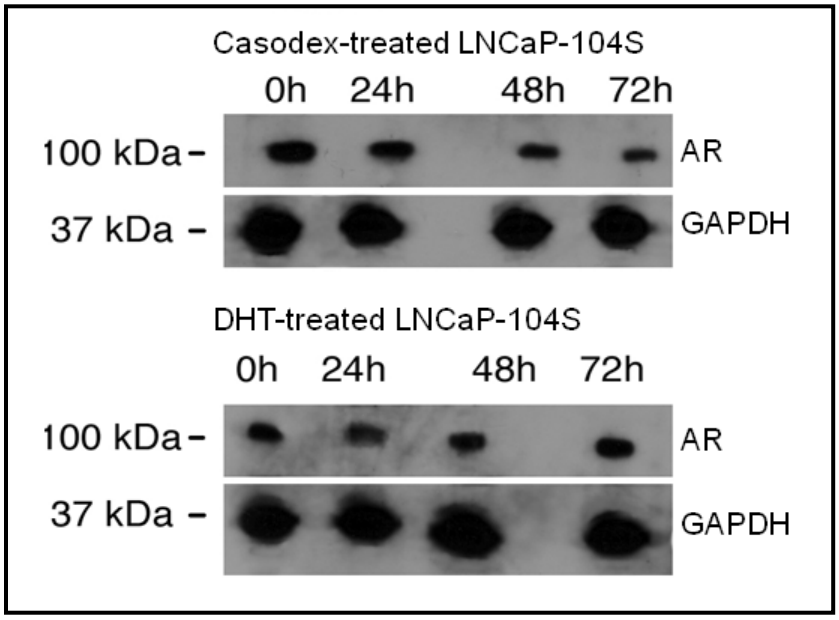

Figure 1) Western blot of androgen receptor (110 kDa) in Casodex-treated androgen sensitive LNCaP-104S cells and in DHT-treated androgen sensitive LNCaP-104S cells at zero hour, 24 hours, 48 hours, and 72 hours timepoints; GAPDH loading control

The Ct results of miRNome screening in five LNCaP-104S treatment groups (zero hour, one week CS-FBS, three weeks CS-FBS, one week CDX, three weeks CDX) and the androgen independent zero hour LNCaP-104R1 group were processed using the ΔΔCt method with LNCaP-104S zero hour as the control group for each, and then compiled into a color-coded table of miRNA expression values, presented as Table 2. The standard rule of thumb that three-fold changes in expression, positive or negative, are to be considered significant miRNA expression changes was used as the initial method of looking at patterns across the five columns of ΔΔCt data. Fold amplification values with green backgrounds are greater than 3.0 and those with red backgrounds are less than -3.0 (corresponding to a threshold of 0.33 for untransformed fold amplification values). A value of 0.00 indicates that no cDNA amplification was detected, either because of extremely low miRNA expression level or an error in amplification.

Table 2a) MiRNome expression screening; 1- 104S 1 wk CSFBS, 2- 104S 3 wk CSFBS, 3- 104S 1 wk CDX, 4- 104S 3 wk CDX, 5- 0 hr 104R; All values are comparisons with 0 hr 104S; Green denotes change > 3 fold; Red denotes change < -3 fold

miRNA	1	2	3	4	5
miR-RC-1	0.00	-4.01	2.02	-2.64	1.60
miR-RC-2	0.00	0.00	0.00	0.00	0.00
miR-RC-3	10.87	10.98	5.39	17.35	21.96
miR-RC-4	3.17	-2.09	-1.01	-1.50	1.58
miR-RC-5	158.80	79.72	0.00	127.61	80.89
miR-RC-6	3.12	-2.49	1.95	-1.63	1.54
miR-RC-7	20.57	21.30	2.43	67.17	0.00
miR-RC-8	3.19	-3.68	-1.04	-1.98	3.10
miR-RC-9	4.94	2.42	2.43	8.02	4.83
miR-RC-10	1.53	-5.95	-1.02	-3.38	1.58
miR-RC-11	9.84	5.06	4.85	7.70	4.89
miR-RC-12	3.21	-61.68	-2.00	-133.31	-2.55
miR-RC-13	0.00	0.00	0.00	0.00	0.00
miR-RC-14	2.56	1.26	1.25	4.06	4.96
miR-RC-15	1.53	-3.21	2.03	-1.59	1.58
miR-RC-16	0.00	0.00	0.00	0.00	0.00
miR-RC-17	-1.28	-3.14	-2.03	-1.59	-1.28
miR-RC-18	0.00	0.00	0.00	0.00	0.00
miR-RC-19	0.00	0.00	0.00	0.00	0.00
miR-RC-20	1.59	-2.28	-1.04	-1.58	-2.54
miR-RC-21	0.00	0.00	0.00	0.00	0.00
miR-RC-22	3.18	-44.81	4.01	-4.92	1.56
miR-RC-23	-1.60	-1.62	-1.64	-1.00	-1.62
miR-RC-24	1.61	-1.14	2.00	1.52	3.20
miR-RC-25	2.42	1.22	1.21	1.95	1.20
miR-RC-26	-2.60	-30.46	-4.05	-3.30	-1.26
miR-RC-27	-2.58	-7.49	-2.09	-4.57	-2.54
miR-RC-28	-3.25	-1.62	-6.76	1.97	-3.23
miR-RC-29	-2.61	-15.50	-2.04	-8.63	1.56
miR-RC-30	-3.25	-1.63	-1.83	-1.02	1.21
miR-RC-31	-1.29	-4.26	-1.04	-3.31	1.56
miR-RC-32	1.23	-3.27	-1.66	1.00	-1.62
miR-RC-33	1.53	-3.04	1.99	-1.60	3.06
miR-RC-34	3.09	-1.82	1.97	-1.11	6.29
miR-RC-35	4.86	1.21	4.85	3.89	0.00
miR-RC-36	3.23	-1.82	2.02	1.22	12.71
miR-RC-37	1.22	2.45	1.19	1.95	2.38
miR-RC-38	-9.37	-7.02	-3.53	-3.23	-4.57
miR-RC-39	-13.05	2.60	1.27	1.09	0.00
miR-RC-40	-2.75	-1.63	-1.68	-3.37	-2.12

miRNA	1	2	3	4	5
miR-RC-41	-2.13	-1.63	0.00	-1.66	-2.03
miR-RC-42	-2.01	-3.19	-3.33	-3.29	-2.15
miR-RC-43	-1.82	-3.23	0.00	-1.60	-1.01
miR-RC-44	1.18	1.27	-1.56	1.24	1.00
miR-RC-45	0.00	0.00	0.00	0.00	0.00
miR-RC-46	-8.56	-6.52	-3.31	1.23	-5.11
miR-RC-47	1.03	-1.63	-1.62	-1.65	-1.04
miR-RC-48	-2.18	-1.62	-3.14	-3.23	-1.03
miR-RC-49	-1.83	-1.58	-1.61	-1.58	-2.05
miR-RC-50	-1.71	-1.64	-1.66	-1.69	-1.05
miR-RC-51	-1.78	0.00	0.00	1.16	3.79
miR-RC-52	3.47	6.00	5.99	6.05	1.23
miR-RC-53	0.00	0.00	0.00	0.00	0.00
miR-RC-54	-1.39	-3.29	2.50	1.20	1.93
miR-RC-55	-2.41	-1.62	-3.32	-3.38	-2.07
miR-RC-56	-1.18	1.22	2.42	2.38	1.90
miR-RC-57	-1.26	-6.12	1.93	1.96	-1.31
miR-RC-58	0.00	0.00	0.00	0.00	3.01
miR-RC-59	-1.08	-3.28	-1.65	-6.65	-1.04
miR-RC-60	1.54	1.27	-1.56	1.25	2.00
miR-RC-61	-2.64	-3.27	-1.62	-1.59	1.05
miR-RC-62	1.00	1.28	2.46	1.21	1.93
miR-RC-63	-2.12	-3.29	-1.62	-13.07	-2.05
miR-RC-64	-1.62	-3.19	-3.24	-3.93	-1.59
miR-RC-65	-1.69	-6.65	-3.18	-3.20	-4.06
miR-RC-66	-2.49	-25.86	0.00	-1.67	-2.08
miR-RC-67	-1.61	-1.56	-3.30	-2.00	-1.60
miR-RC-68	-1.22	1.23	1.22	-1.67	-2.11
miR-RC-69	1.22	-1.59	-1.63	-1.61	-1.00
miR-RC-70	-2.81	-1.64	-1.63	-1.65	-2.04
miR-RC-71	1.37	2.51	2.57	2.50	2.01
miR-RC-72	-1.10	-1.63	-1.61	1.19	-2.05
miR-RC-73	0.00	0.00	0.00	0.00	0.00
miR-RC-74	1.65	-1.61	2.50	1.24	-4.11
miR-RC-75	-1.29	-13.58	-1.03	-6.37	6.26
miR-RC-76	1.23	-1.11	4.76	3.69	4.78
miR-RC-77	0.00	0.00	0.00	0.00	0.00
miR-RC-78	-4.72	-12.88	-6.52	-13.61	1.90
miR-RC-79	-1.50	1.26	1.28	-1.61	-2.04
miR-RC-80	-5.07	-3.31	-3.18	-3.27	-4.10

Table 2b) MiRNome expression screening; 1- 104S 1 wk CSFBS, 2- 104S 3 wk CSFBS, 3- 104S 1 wk CDX, 4- 104S 3 wk CDX, 5- 0 hr 104R; All values are comparisons with 0 hr 104S; Green denotes change > 3 fold; Red denotes change < -3 fold

miRNA	1	2	3	4	5
miR-RC-81	-2.80	-3.17	-3.14	-3.18	-4.13
miR-RC-82	-1.06	2.45	-1.62	1.22	-2.05
miR-RC-83	1.16	1.25	1.24	1.21	1.92
miR-RC-84	-1.10	-1.66	-1.64	-1.65	-2.12
miR-RC-85	1.95	2.45	2.52	1.21	-1.03
miR-RC-86	0.00	0.00	0.00	0.00	0.00
miR-RC-87	-1.74	-1.58	-1.57	-1.63	-3.97
miR-RC-88	1.11	1.25	-1.60	1.22	1.96
miR-RC-89	1.59	-1.58	2.53	1.23	0.00
miR-RC-90	-2.08	-1.61	1.24	-1.64	-2.06
miR-RC-91	1.60	1.24	2.46	1.23	1.96
miR-RC-92	1.68	1.24	2.51	2.42	1.92
miR-RC-93	1.45	4.98	2.52	4.94	2.00
miR-RC-94	-1.39	-1.54	2.56	-1.62	2.04
miR-RC-95	3.09	-1.15	1.92	-1.21	3.12
miR-RC-96	1.52	-1.67	-1.02	0.00	-1.30
miR-RC-97	3.14	-1.37	1.94	1.16	-2.56
miR-RC-98	1.19	-1.67	1.16	-1.00	-1.67
miR-RC-99	1.26	1.26	1.21	2.04	20.16
miR-RC-100	-1.28	-3.99	-1.03	-1.64	-2.56
miR-RC-101	5.02	2.50	4.90	16.20	2.44
miR-RC-102	-2.32	-1.60	1.23	-1.63	-1.02
miR-RC-103	-1.36	-1.61	0.00	-1.62	0.00
miR-RC-104	-31.85	0.00	1.47	2.89	2.25
miR-RC-105	1.90	-1.64	1.23	-1.66	3.90
miR-RC-106	3.21	2.48	9.73	9.76	15.79
miR-RC-107	2.02	-1.65	-1.86	-1.60	1.96
miR-RC-108	-2.22	-1.64	-1.62	-1.62	-1.01
miR-RC-109	-2.89	-6.45	-1.61	-3.26	-2.01
miR-RC-110	-1.34	-1.56	-3.08	-1.59	-1.02
miR-RC-111	1.58	-1.61	-1.57	-1.64	-1.03
miR-RC-112	-2.50	-3.24	-3.29	-1.64	-2.04
miR-RC-113	-3.55	-3.17	-1.63	-1.62	-2.02
miR-RC-114	-1.16	-1.60	1.27	-1.72	-2.04
miR-RC-115	-1.38	-3.22	-1.62	-1.61	-2.03
miR-RC-116	1.58	-3.07	-2.06	-2.60	1.56
miR-RC-117	-2.60	-3.13	-3.19	-3.22	-4.02
miR-RC-118	-5.79	-3.25	-3.31	-3.46	-4.24
miR-RC-119	-2.18	-3.28	-1.58	-3.28	-2.07
miR-RC-120	-2.49	-3.63	-2.03	-2.37	-1.25

miRNA	1	2	3	4	5
miR-RC-121	-2.34	-1.57	-3.23	-1.56	-1.02
miR-RC-122	0.00	0.00	0.00	0.00	0.00
miR-RC-123	0.00	0.00	0.00	0.00	0.00
miR-RC-124	0.00	0.00	0.00	0.00	0.00
miR-RC-125	-1.26	-2.70	-4.14	-2.43	1.56
miR-RC-126	-1.65	-1.65	1.26	-1.62	-1.02
miR-RC-127	-1.82	0.00	-1.64	-1.61	-2.04
miR-RC-128	1.22	1.23	2.40	1.16	1.90
miR-RC-129	-1.97	1.22	1.23	-1.65	1.87
miR-RC-130	-1.59	-1.64	-1.66	1.21	-1.05
miR-RC-131	-2.34	1.26	-6.55	-1.66	-1.04
miR-RC-132	1.05	1.23	-1.63	1.20	-1.06
miR-RC-133	1.53	0.00	2.49	2.39	1.94
miR-RC-134	-4.24	-6.59	-3.19	-1.67	-4.25
miR-RC-135	-3.40	1.23	2.37	2.40	-1.03
miR-RC-136	0.00	0.00	0.00	0.00	0.00
miR-RC-137	-2.44	-3.30	-1.56	-3.26	-2.02
miR-RC-138	-1.75	-1.69	-6.84	-3.39	-4.29
miR-RC-139	-1.39	-1.65	1.22	-1.69	-2.08
miR-RC-140	-4.08	-1.65	-1.69	-6.77	-4.23
miR-RC-141	-1.26	-60.08	-1.04	-2.29	1.56
miR-RC-142	-2.97	-6.46	-3.29	-6.60	-4.15
miR-RC-143	-1.65	1.26	1.23	1.22	-1.04
miR-RC-144	-1.27	-4.47	-3.98	-2.41	-1.27
miR-RC-145	-2.56	-3.15	-3.17	-3.17	-2.00
miR-RC-146	0.00	0.00	0.00	0.00	0.00
miR-RC-147	2.37	2.62	2.68	1.31	2.12
miR-RC-148	-3.65	-6.42	-1.62	-6.86	-2.07
miR-RC-149	-1.36	-1.62	-1.60	1.22	1.97
miR-RC-150	-3.77	-1.56	-6.25	1.27	-2.05
miR-RC-151	-16.43	0.00	0.00	0.00	0.00
miR-RC-152	-2.66	-1.63	-1.65	-1.67	-1.01
miR-RC-153	-2.54	-3.32	-1.63	-3.46	-1.05
miR-RC-154	-3.85	-6.43	1.26	-3.35	-4.14
miR-RC-155	3.58	1.22	5.07	2.40	1.91
miR-RC-156	1.63	1.23	0.00	1.21	1.91
miR-RC-157	-4.60	-3.22	-3.20	1.24	-3.89
miR-RC-158	-1.28	-34.03	-2.27	0.00	3.09
miR-RC-159	9.67	4.80	4.92	7.85	10.00
miR-RC-160	-1.25	-3.68	-1.97	-5.04	1.58

Table 2c) MiRNome expression screening; 1- 104S 1 wk CSFBS, 2- 104S 3 wk CSFBS, 3- 104S 1 wk CDX, 4- 104S 3 wk CDX, 5- 0 hr 104R; All values are comparisons with 0 hr 104S; Green denotes change > 3 fold; Red denotes change < -3 fold

miRNA	1	2	3	4	5
miR-RC-161	1.24	-6.48	1.24	1.02	1.26
miR-RC-162	-1.29	-3.10	-1.03	-1.64	-1.29
miR-RC-163	1.25	2.48	4.97	4.04	2.47
miR-RC-164	-3.91	-6.39	-3.18	-1.62	-4.41
miR-RC-165	1.64	1.25	1.26	2.42	1.95
miR-RC-166	1.23	-3.20	-3.20	-3.25	1.94
miR-RC-167	-1.90	0.00	2.76	1.18	-1.07
miR-RC-168	-1.22	-5.39	-1.01	-6.54	-1.25
miR-RC-169	1.58	-9.83	1.99	-2.25	1.57
miR-RC-170	-1.27	-6.58	1.99	-4.86	-1.30
miR-RC-171	1.59	-2.67	2.01	-2.85	3.12
miR-RC-172	1.28	1.27	1.27	2.05	1.27
miR-RC-173	-2.61	-16.35	-1.03	-8.45	1.55
miR-RC-174	1.26	-1.61	-1.64	1.00	1.22
miR-RC-175	6.68	-18.51	4.18	2.35	3.36
miR-RC-176	-3.16	-3.56	-1.78	-1.10	-1.61
miR-RC-177	1.59	-2.88	1.02	-1.53	-2.50
miR-RC-178	-1.28	-12.56	-2.04	-6.93	3.13
miR-RC-179	-1.66	-1.66	-3.29	-1.05	-3.39
miR-RC-180	-1.66	1.26	-1.64	-1.03	-1.63
miR-RC-181	1.58	-1.99	1.95	-3.03	1.59
miR-RC-182	-1.29	-4.74	1.93	-2.48	-1.27
miR-RC-183	1.55	-1.55	2.01	-1.29	3.13
miR-RC-184	1.60	-1.65	-1.01	1.05	3.11
miR-RC-185	1.57	-7.84	-2.02	-6.72	1.58
miR-RC-186	4.89	2.53	1.24	4.05	2.43
miR-RC-187	0.00	0.00	0.00	0.00	0.00
miR-RC-188	-1.20	0.00	1.92	0.00	1.58
miR-RC-189	3.08	-1.15	3.86	2.06	3.13
miR-RC-190	1.29	1.27	-1.63	2.03	1.26
miR-RC-191	0.00	0.00	0.00	0.00	0.00
miR-RC-192	0.00	0.00	0.00	0.00	0.00
miR-RC-193	0.00	0.00	0.00	0.00	0.00
miR-RC-194	-1.55	1.24	0.00	0.00	2.54
miR-RC-195	1.01	-3.24	1.21	-3.28	-4.30
miR-RC-196	-1.58	-1.56	-3.03	-3.23	-2.01
miR-RC-197	51.04	45.91	30.99	36.06	-1.26
miR-RC-198	-1.65	-1.64	-1.08	1.91	1.22
miR-RC-199	1.61	-3.26	-8.01	-7.00	-1.27
miR-RC-200	1.54	-2.87	-2.08	0.00	-2.56

miRNA	1	2	3	4	5
miR-RC-201	1.56	-4.65	-2.01	-2.37	-1.25
miR-RC-202	-1.87	-1.63	-3.22	-3.27	-2.06
miR-RC-203	-1.07	-1.64	-1.62	-1.65	-1.04
miR-RC-204	1.54	-3.02	1.96	-1.33	3.02
miR-RC-205	1.57	-2.05	-1.02	-1.15	1.56
miR-RC-206	1.23	1.25	-1.65	2.06	1.23
miR-RC-207	3.16	-2.02	3.97	-2.67	3.20
miR-RC-208	-6.60	-6.38	-27.53	-4.04	-6.53
miR-RC-209	-1.26	-9.44	-1.04	-10.36	-1.28
miR-RC-210	-1.63	-1.61	-1.63	-1.02	-1.65
miR-RC-211	1.55	-9.08	-2.00	-1.52	1.55
miR-RC-212	-1.65	-3.26	-3.30	-2.08	-1.62
miR-RC-213	-1.24	-3.24	1.01	-1.55	1.59
miR-RC-214	1.56	1.02	1.96	1.92	3.11
miR-RC-215	1.57	-1.81	1.96	-1.48	-1.27
miR-RC-216	-2.59	-25.19	0.00	-35.10	-3.04
miR-RC-217	-1.89	-1.58	-3.18	-1.65	-2.03
miR-RC-218	-1.84	-3.25	-3.22	-3.29	-2.15
miR-RC-219	-2.27	-3.23	-3.23	-1.60	-2.02
miR-RC-220	1.60	-1.90	3.91	-15.44	12.73
miR-RC-221	1.24	1.15	4.82	3.46	0.00
miR-RC-222	3.14	-8.15	-1.02	0.00	-1.25
miR-RC-223	-7.27	-3.18	-1.51	0.00	-1.59
miR-RC-224	1.57	-1.05	1.01	1.24	1.59
miR-RC-225	1.23	-1.59	-1.61	-1.00	-1.60
miR-RC-226	-1.27	-10.96	-2.07	-6.37	1.57
miR-RC-227	-1.70	-6.64	-3.36	-8.50	2.39
miR-RC-228	-1.27	-2.39	-1.03	-1.85	1.57
miR-RC-229	1.20	1.22	2.40	1.90	2.40
miR-RC-230	1.23	-1.59	1.23	-2.01	2.41
miR-RC-231	0.00	0.00	0.00	0.00	0.00
miR-RC-232	-1.64	-3.24	-1.62	-4.12	2.44
miR-RC-233	0.00	0.00	0.00	0.00	0.00
miR-RC-234	1.23	-4.01	-1.66	-1.37	4.79
miR-RC-235	0.00	-3.32	-1.71	-2.09	-1.71
miR-RC-236	-1.29	-2.39	-2.01	-2.57	-1.29
miR-RC-237	-1.28	-2.45	-1.00	-1.27	1.57
miR-RC-238	-1.62	-1.60	-6.74	2.00	-3.27
miR-RC-239	1.53	-2.22	-2.03	-1.35	1.56
miR-RC-240	3.21	-2.39	1.92	-1.13	3.13

Table 2d) MiRNome expression screening; 1- 104S 1 wk CSFBS, 2- 104S 3 wk CSFBS, 3- 104S 1 wk CDX, 4- 104S 3 wk CDX, 5- 0 hr 104R; All values are comparisons with 0 hr 104S; Green denotes change > 3 fold; Red denotes change < -3 fold

miRNA	1	2	3	4	5
miR-RC-241	19.67	5.07	19.95	8.21	10.18
miR-RC-242	-1.64	-3.35	-1.62	-1.62	-2.09
miR-RC-243	-2.14	-1.63	-3.39	-1.64	-2.08
miR-RC-244	0.00	0.00	0.00	0.00	0.00
miR-RC-245	0.00	-5.87	0.00	0.00	3.13
miR-RC-246	2.42	1.21	1.23	2.01	1.21
miR-RC-247	-2.68	-28.12	-1.22	-101.37	0.00
miR-RC-248	1.59	-2.30	1.00	-1.29	3.14
miR-RC-249	2.53	1.28	1.27	2.02	2.56
miR-RC-250	1.56	-1.69	-1.02	-1.13	1.56
miR-RC-251	-1.61	1.19	0.00	0.00	2.32
miR-RC-252	-2.55	-5.04	-4.14	-3.82	-2.62
miR-RC-253	1.24	1.22	-1.62	-1.02	1.26
miR-RC-254	1.60	1.38	-1.00	2.92	1.54
miR-RC-255	-1.29	-3.21	-2.02	-2.40	-1.28
miR-RC-256	-2.54	-64.36	-4.05	-42.30	1.56
miR-RC-257	0.00	0.00	0.00	0.00	0.00
miR-RC-258	-2.56	-66.91	-2.00	-65.65	1.54
miR-RC-259	-1.65	0.00	-3.34	-2.06	-3.26
miR-RC-260	0.00	0.00	0.00	0.00	0.00
miR-RC-261	-1.81	-1.63	-1.66	-3.41	-2.07
miR-RC-262	1.03	-3.24	-1.65	-3.29	-2.13
miR-RC-263	-1.64	-1.66	-1.67	-1.03	-1.64
miR-RC-264	-2.59	0.00	1.60	0.00	-1.46
miR-RC-265	-1.25	-1.62	1.03	1.18	1.60
miR-RC-266	2.40	2.41	2.40	1.91	1.19
miR-RC-267	-1.39	-1.69	-1.65	-3.29	-2.05
miR-RC-268	-4.01	-3.37	-6.50	-1.66	-1.99
miR-RC-269	-1.60	-1.65	-1.64	1.01	-1.65
miR-RC-270	-2.17	-1.62	-3.26	-3.48	-2.12
miR-RC-271	-1.35	-1.59	0.00	-1.70	2.01
miR-RC-272	-1.42	-104.71	1.24	-3.35	-1.03
miR-RC-273	-1.59	-3.21	-3.19	-2.03	-1.62
miR-RC-274	-2.98	-1.59	-3.23	-3.35	-2.20
miR-RC-275	-1.69	-3.31	-3.39	-2.06	-1.68
miR-RC-276	-2.55	-7.96	-1.98	-6.65	-1.27
miR-RC-277	-1.64	-1.65	-1.66	-2.05	2.43
miR-RC-278	-1.28	-2.27	-1.00	-1.54	1.60
miR-RC-279	-1.25	-4.39	-1.98	-3.01	-1.28
miR-RC-280	1.56	-1.63	-1.00	-1.73	1.57

miRNA	1	2	3	4	5
miR-RC-281	1.23	1.25	2.53	4.01	2.45
miR-RC-282	1.57	-3.36	-1.03	-1.27	1.55
miR-RC-283	1.26	-3.17	-6.31	2.04	-1.60
miR-RC-284	-1.28	-5.30	2.02	-3.12	-1.30
miR-RC-285	1.22	-1.62	1.22	-1.02	-1.61
miR-RC-286	1.56	-3.31	1.99	-4.61	1.55
miR-RC-287	-1.63	-1.66	-3.30	1.92	-1.63
miR-RC-288	1.58	-7.15	1.95	-2.46	6.27
miR-RC-289	-1.65	-3.28	-3.42	-4.18	1.23
miR-RC-290	-1.28	-12.29	-2.08	-7.80	-1.29
miR-RC-291	-2.44	-3.25	-3.26	-102.85	-2.09
miR-RC-292	-1.24	2.45	1.26	2.42	1.98
miR-RC-293	-3.84	-1.61	-1.65	1.19	-1.03
miR-RC-294	1.35	2.51	1.24	2.48	1.93
miR-RC-295	1.15	-1.62	-1.67	1.24	-1.05
miR-RC-296	-3.04	-3.29	-6.54	-3.26	-4.16
miR-RC-297	-1.15	1.23	2.00	1.21	-2.03
miR-RC-298	2.37	2.49	2.49	2.54	3.94
miR-RC-299	3.16	-1.33	1.97	1.15	3.16
miR-RC-300	1.14	-18.39	5.80	0.00	9.38
miR-RC-301	1.59	-1.46	1.95	-79.81	-1.29
miR-RC-302	-1.21	0.00	2.11	-39.31	0.00
miR-RC-303	-1.30	-25.58	1.74	-16.63	1.41
miR-RC-304	-1.27	-21.14	-2.04	-10.39	1.58
miR-RC-305	-6.51	-1.60	-3.91	-1.02	-3.50
miR-RC-306	-1.29	-14.32	-2.08	-8.84	1.56
miR-RC-307	1.22	-1.60	-1.64	1.96	1.21
miR-RC-308	0.00	0.00	0.00	0.00	0.00
miR-RC-309	1.56	-1.45	-1.02	-1.08	-80.79
miR-RC-310	1.21	1.22	-1.67	1.96	-1.65
miR-RC-311	1.58	-2.59	1.96	-1.51	-10.38
miR-RC-312	-1.61	-1.60	-1.66	2.00	-1.59
miR-RC-313	1.55	-1.58	1.93	1.12	3.14
miR-RC-314	2.45	1.25	1.21	1.99	2.46
miR-RC-315	-1.21	-4.70	-1.94	-2.42	3.22
miR-RC-316	0.00	0.00	1.53	0.00	0.00
miR-RC-317	-1.27	-3.29	-2.02	-2.76	-1.26
miR-RC-318	-1.26	-14.36	-4.02	-8.76	-2.52
miR-RC-319	-1.26	-3.42	-2.00	-2.47	1.62
miR-RC-320	2.47	-1.61	-1.81	-1.03	4.98

Table 2e) MiRNome expression screening; 1- 104S 1 wk CSFBS, 2- 104S 3 wk CSFBS, 3- 104S 1 wk CDX, 4- 104S 3 wk CDX, 5- 0 hr 104R; All values are comparisons with 0 hr 104S; Green denotes change > 3 fold; Red denotes change < -3 fold

miRNA	1	2	3	4	5
miR-RC-321	0.00	0.00	0.00	0.00	0.00
miR-RC-322	0.00	0.00	0.00	0.00	0.00
miR-RC-323	0.00	0.00	0.00	0.00	0.00
miR-RC-324	1.56	-4.92	-1.98	-3.38	-1.27
miR-RC-325	-1.28	-7.70	-3.99	-3.96	-5.04
miR-RC-326	4.24	-1.13	5.37	-4.46	0.00
miR-RC-327	-2.56	-30.40	-2.06	-14.66	1.56
miR-RC-328	2.52	-3.17	2.45	-3.98	4.98
miR-RC-329	-1.28	-16.12	-2.06	-48.36	1.57
miR-RC-330	9.77	4.85	4.95	15.70	0.00
miR-RC-331	3.23	-1.11	2.01	1.58	3.15
miR-RC-332	5.07	5.02	4.98	7.87	0.00
miR-RC-333	-1.27	-8.75	-1.04	-1.81	-1.28
miR-RC-334	-1.24	-12.16	-2.02	-8.61	-1.25
miR-RC-335	-1.26	1.27	-1.61	-1.70	1.97
miR-RC-336	-1.32	-3.28	-1.62	-1.65	-2.02
miR-RC-337	-2.57	-4.08	-1.97	-4.99	-1.23
miR-RC-338	1.57	-1.76	-1.01	-2.04	1.57
miR-RC-339	-1.62	-3.24	-3.32	-2.03	-1.62
miR-RC-340	3.43	1.37	2.06	-3.46	1.60
miR-RC-341	1.57	-2.77	-1.02	-1.22	1.56
miR-RC-342	1.59	-1.62	3.92	-1.75	-1.31
miR-RC-343	1.56	-20.97	3.90	-41.27	6.00
miR-RC-344	0.00	0.00	0.00	0.00	0.00
miR-RC-345	-1.64	-3.28	-3.28	1.96	-3.24
miR-RC-346	-3.23	-3.22	-1.65	-1.01	-3.26
miR-RC-347	1.56	-2.13	-1.02	-1.91	-1.30
miR-RC-348	1.55	-1.97	1.97	-1.78	1.56
miR-RC-349	-1.28	-7.48	-1.00	-7.29	1.57
miR-RC-350	6.29	3.28	3.90	3.37	6.35
miR-RC-351	20.22	40.40	9.73	65.36	0.00
miR-RC-352	3.66	-5.21	2.22	-2.39	1.63
miR-RC-353	1.57	-1.23	1.02	-1.21	1.56
miR-RC-354	1.56	-2.45	-2.04	-2.09	-1.30
miR-RC-355	-1.27	-1.82	-2.01	-1.95	-1.29
miR-RC-356	1.19	2.43	2.37	1.91	1.19
miR-RC-357	3.16	3.59	-1.02	0.00	-1.26
miR-RC-358	2.47	2.53	2.45	4.02	1.20
miR-RC-359	1.60	-1.27	1.96	1.06	3.13
miR-RC-360	1.23	1.22	-1.67	1.96	1.18

miRNA	1	2	3	4	5
miR-RC-361	-1.27	-2.82	-2.03	-1.09	1.57
miR-RC-362	-3.23	1.23	-1.63	1.97	2.43
miR-RC-363	1.57	0.00	1.96	-1.88	1.58
miR-RC-364	2.53	1.27	0.00	4.00	1.25
miR-RC-365	1.56	-2.51	-1.02	-1.92	1.60
miR-RC-366	4.98	2.49	2.44	2.02	5.06
miR-RC-367	3.12	1.04	1.98	1.47	3.11
miR-RC-368	-3.28	-1.65	0.00	1.61	1.20
miR-RC-369	1.22	2.48	1.21	1.96	1.20
miR-RC-370	1.60	-2.88	1.00	-1.82	1.59
miR-RC-371	2.47	2.45	1.24	2.01	-1.62
miR-RC-372	1.53	-3.06	1.99	-2.32	3.16
miR-RC-373	1.22	1.25	1.23	1.98	-1.64
miR-RC-374	-1.70	0.00	0.00	0.00	2.28
miR-RC-375	25.28	-21.51	3.96	1.49	24.97
miR-RC-376	-1.59	-3.18	-1.63	-1.00	-3.17
miR-RC-377	1.59	-3.01	-1.01	-2.20	-1.30
miR-RC-378	1.25	-1.56	-1.59	-4.04	-1.62
miR-RC-379	-1.26	-7.46	1.01	-6.61	1.59
miR-RC-380	-1.62	-1.60	-1.64	1.98	1.23
miR-RC-381	1.61	-1.52	-1.01	1.07	1.57
miR-RC-382	1.58	1.01	1.98	1.83	3.11
miR-RC-383	1.52	1.26	-1.03	2.12	1.47
miR-RC-384	1.58	-6.08	-1.99	-4.63	1.56
miR-RC-385	1.59	-3.67	-2.03	-1.68	-1.27
miR-RC-386	-1.28	-1.62	-1.07	1.10	1.48
miR-RC-387	3.22	-1.42	1.92	-3.27	3.17
miR-RC-388	3.43	-1.01	2.04	1.89	1.54
miR-RC-389	3.09	2.47	1.96	2.64	3.15
miR-RC-390	1.21	1.25	2.44	1.95	-1.64
miR-RC-391	3.14	1.08	-1.02	1.03	1.61
miR-RC-392	2.49	1.29	-3.22	4.03	-1.61
miR-RC-393	1.24	1.25	1.24	3.99	1.24
miR-RC-394	1.55	-1.14	1.94	1.24	1.57
miR-RC-395	2.52	1.25	1.23	4.06	0.00
miR-RC-396	-1.25	-4.13	1.01	-8.90	-1.23
miR-RC-397	-1.27	-4.29	-2.01	-4.22	-1.28
miR-RC-398	3.09	-2.91	-1.04	-2.86	-1.26
miR-RC-399	-1.26	-1.39	1.99	-3.90	3.16
miR-RC-400	5.00	5.05	2.47	8.11	2.38

Table 2f) MiRNome expression screening; 1- 104S 1 wk CSFBS, 2- 104S 3 wk CSFBS, 3- 104S 1 wk CDX, 4- 104S 3 wk CDX, 5- 0 hr 104R; All values are comparisons with 0 hr 104S; Green denotes change > 3 fold; Red denotes change < -3 fold

miRNA	1	2	3	4	5
miR-RC-401	-5.40	0.00	0.00	-88.24	0.00
miR-RC-402	2.48	1.29	1.18	4.03	9.88
miR-RC-403	1.57	-1.15	1.99	-1.03	-2.52
miR-RC-404	4.95	2.52	1.22	3.92	4.89
miR-RC-405	-1.26	-2.73	2.00	-1.79	-1.25
miR-RC-406	1.28	1.26	1.22	1.96	1.23
miR-RC-407	-1.29	-11.58	1.00	-78.03	3.15
miR-RC-408	0.00	0.00	0.00	0.00	0.00
miR-RC-409	1.57	-1.89	-1.05	-1.16	3.20
miR-RC-410	1.23	-1.64	-1.64	-1.02	2.41
miR-RC-411	1.57	-1.81	1.96	-1.19	1.56
miR-RC-412	-7.86	2.52	1.22	1.99	2.46
miR-RC-413	-2.57	-6.52	-2.08	-4.36	-1.28
miR-RC-414	-3.33	-6.41	0.00	-1.01	-3.46
miR-RC-415	0.00	0.00	0.00	0.00	0.00
miR-RC-416	1.55	-1.48	-1.03	-1.01	3.17
miR-RC-417	-1.61	-1.59	-3.27	1.00	-1.60
miR-RC-418	1.59	-3.03	-1.03	-1.87	3.12
miR-RC-419	80.56	40.90	81.48	128.42	162.70
miR-RC-420	-1.28	-7.53	-2.04	-1.25	-1.30
miR-RC-421	1.20	2.47	1.16	1.76	0.00
miR-RC-422	3.11	-10.14	1.89	-2.78	6.18
miR-RC-423	0.00	0.00	0.00	0.00	0.00
miR-RC-424	-1.29	-3.22	-1.03	-4.49	-1.29
miR-RC-425	1.59	-1.96	1.01	-2.16	1.56
miR-RC-426	1.59	-2.89	-1.01	-2.58	1.56
miR-RC-427	-2.57	-4.71	-1.01	-2.37	1.58
miR-RC-428	-1.27	-2.58	-1.01	-1.88	1.57
miR-RC-429	-1.28	-3.47	-2.05	-3.41	-1.29
miR-RC-430	-1.27	-13.31	-3.95	-13.29	-1.31
miR-RC-431	-2.53	-4.09	-2.03	-2.34	-2.57
miR-RC-432	-2.59	-4.00	-4.17	-2.05	1.54
miR-RC-433	-1.27	-5.84	-2.02	-3.98	-1.25
miR-RC-434	1.59	-1.83	-2.00	-2.14	-1.25
miR-RC-435	1.54	-4.10	1.98	-3.18	-1.27
miR-RC-436	1.56	1.10	-1.01	1.12	1.57
miR-RC-437	1.58	-5.45	-1.02	-2.85	-1.25
miR-RC-438	1.59	-1.21	1.99	-1.20	-1.29
miR-RC-439	-1.26	-2.46	-1.99	-6.21	-2.60
miR-RC-440	1.58	-2.62	2.01	-1.55	6.34

miRNA	1	2	3	4	5
miR-RC-441	-1.51	-3.24	-1.60	1.03	1.28
miR-RC-442	1.55	-3.10	1.00	-77.45	-1.29
miR-RC-443	3.14	1.35	1.82	1.98	3.18
miR-RC-444	6.31	-1.75	1.96	1.95	6.27
miR-RC-445	1.59	-2.09	1.00	-2.47	-1.26
miR-RC-446	-1.27	-5.05	-2.05	-6.18	-1.28
miR-RC-447	-1.27	-6.31	-2.04	-6.16	-1.25
miR-RC-448	-1.24	-4.38	-4.07	-9.97	3.17
miR-RC-449	2.34	-1.66	-1.69	1.88	1.15
miR-RC-450	-2.49	-7.96	-2.08	-7.46	3.10
miR-RC-451	2.49	1.21	1.22	2.02	1.24
miR-RC-452	13.34	-5.23	16.51	-3.11	25.63
miR-RC-453	1.20	-3.24	2.44	2.03	1.21
miR-RC-454	1.55	-3.49	-1.05	-2.75	1.55
miR-RC-455	-1.27	-1.63	3.95	3.54	6.27
miR-RC-456	-1.24	-3.93	-1.98	-2.40	-2.53
miR-RC-457	1.61	-3.29	1.01	-3.36	1.60
miR-RC-458	1.58	-1.78	-1.04	-1.03	1.53
miR-RC-459	1.23	-1.59	1.24	1.01	1.26
miR-RC-460	3.09	-4.05	-1.02	-1.34	1.57
miR-RC-461	2.43	2.52	2.48	7.76	2.53
miR-RC-462	-1.28	-4.87	-4.05	-7.71	-1.27
miR-RC-463	6.20	-1.56	1.98	-2.79	-1.29
miR-RC-464	3.13	1.03	-1.01	2.24	3.05
miR-RC-465	3.18	-2.18	4.06	-2.57	6.31
miR-RC-466	1.58	-3.84	-1.02	-1.79	3.15
miR-RC-467	-1.27	-3.59	1.96	-2.11	1.55
miR-RC-468	-5.08	-16.15	-8.22	-13.98	-20.17
miR-RC-469	0.00	0.00	0.00	0.00	0.00
miR-RC-470	3.22	-4.20	1.96	-1.82	3.22
miR-RC-471	1.18	1.23	1.25	2.00	1.26
miR-RC-472	-1.28	-4.43	-2.20	-3.25	-1.27
miR-RC-473	2.50	2.56	5.16	4.06	5.10
miR-RC-474	0.00	0.00	0.00	0.00	0.00
miR-RC-475	0.00	0.00	0.00	0.00	0.00
miR-RC-476	-1.27	-1.91	-1.01	-1.42	1.57
miR-RC-477	-1.33	-19.83	3.88	-1.22	1.45
miR-RC-478	1.52	-4.48	-2.07	-3.25	1.55
miR-RC-479	1.62	-1.06	4.11	1.11	6.49
miR-RC-480	-1.29	-6.98	-4.02	-3.21	-1.29

Table 2g) MiRNome expression screening; 1- 104S 1 wk CSFBS, 2- 104S 3 wk CSFBS, 3- 104S 1 wk CDX, 4- 104S 3 wk CDX, 5- 0 hr 104R; All values are comparisons with 0 hr 104S; Green denotes change > 3 fold; Red denotes change < -3 fold

miRNA	1	2	3	4	5
miR-RC-481	1.22	1.24	1.21	1.92	1.22
miR-RC-482	0.00	0.00	0.00	0.00	0.00
miR-RC-483	0.00	0.00	0.00	0.00	0.00
miR-RC-484	1.55	-2.36	1.97	-1.18	6.41
miR-RC-485	0.00	0.00	0.00	0.00	0.00
miR-RC-486	-1.29	-16.27	-1.01	-5.13	-5.23
miR-RC-487	-1.29	1.71	7.93	1.02	1.54
miR-RC-488	-1.60	1.29	1.22	0.00	0.00
miR-RC-489	0.00	0.00	0.00	0.00	0.00
miR-RC-490	1.60	0.00	-1.10	1.01	-1.37
miR-RC-491	-1.35	-6.03	1.92	-3.57	3.47
miR-RC-492	-1.62	-3.23	1.23	-1.02	-1.49
miR-RC-493	-1.28	-5.22	1.00	-2.38	1.60
miR-RC-494	-1.63	-3.27	-1.66	-1.04	-1.65
miR-RC-495	1.52	-1.70	3.79	-2.44	1.53
miR-RC-496	-3.32	-6.73	-3.29	0.00	-3.51
miR-RC-497	1.57	-2.44	2.02	-4.88	1.58
miR-RC-498	-3.19	-1.58	-1.57	1.01	2.48
miR-RC-499	1.53	-2.94	-1.99	-1.18	1.58
miR-RC-500	-1.22	-3.04	-4.18	-1.60	-1.26
miR-RC-501	-1.29	-3.14	-2.07	-1.57	-1.30
miR-RC-502	0.00	0.00	0.00	0.00	0.00
miR-RC-503	-1.27	-1.47	1.00	1.16	1.57
miR-RC-504	-1.25	-1.39	1.01	-1.25	3.23
miR-RC-505	1.59	-1.72	-2.02	-1.21	1.58
miR-RC-506	3.23	-1.18	4.00	1.55	-1.34
miR-RC-507	-6.45	-1.57	-3.23	-1.01	-1.68
miR-RC-508	-1.25	1.05	-2.02	1.07	-1.24
miR-RC-509	-1.28	-2.44	1.95	1.17	1.53
miR-RC-510	-2.57	-9.99	-4.08	-9.40	-2.55
miR-RC-511	1.55	-1.65	2.05	-1.78	1.62
miR-RC-512	-1.23	-3.23	-1.97	-2.74	1.60
miR-RC-513	1.58	1.31	1.01	1.92	3.18
miR-RC-514	-1.64	-3.31	-13.08	-2.03	1.22
miR-RC-515	1.59	-1.89	-1.02	-1.08	3.15
miR-RC-516	-1.63	-1.63	-3.33	-1.03	-1.67
miR-RC-517	1.60	-2.70	2.09	-1.66	-159.95
miR-RC-518	-2.62	-7.35	-4.13	-6.61	-1.28
miR-RC-519	-1.66	-1.66	-3.35	-1.03	1.21
miR-RC-520	-2.63	-4.72	-2.06	-3.83	-1.28

miRNA	1	2	3	4	5
miR-RC-521	1.23	-1.62	-1.63	-1.00	-3.23
miR-RC-522	-1.28	-3.08	-4.01	-3.08	1.54
miR-RC-523	-2.54	-28.82	-4.14	-2.36	-2.53
miR-RC-524	1.55	-3.36	2.00	-2.07	1.61
miR-RC-525	-2.55	-3.61	-1.04	-3.15	-2.62
miR-RC-526	-1.63	0.00	-3.32	-1.02	-1.64
miR-RC-527	-1.27	-9.13	1.01	-18.34	-1.26
miR-RC-528	-1.30	-8.27	1.77	-44.62	-1.37
miR-RC-529	-1.22	-103.35	3.89	-61.45	1.52
miR-RC-530	0.00	1.59	2.24	-3.17	1.67
miR-RC-531	-1.28	-1.88	-4.12	-3.41	1.59
miR-RC-532	-3.24	-3.29	-1.83	-1.06	1.22
miR-RC-533	-1.30	-3.10	1.93	1.17	1.57
miR-RC-534	-1.27	-8.03	-1.02	-7.79	3.12
miR-RC-535	2.47	2.50	5.05	8.12	2.54
miR-RC-536	6.29	1.80	1.99	2.12	3.13
miR-RC-537	3.29	-3.15	16.51	-3.09	3.19
miR-RC-538	-1.29	-5.15	-16.35	-8.60	-2.55
miR-RC-539	-1.29	-2.32	-1.05	-1.52	-1.33
miR-RC-540	-1.26	-4.17	1.01	-3.52	-1.26
miR-RC-541	-1.31	-2.93	-4.03	-1.70	-1.29
miR-RC-542	1.57	1.18	1.89	1.27	1.55
miR-RC-543	-1.31	-1.71	-1.01	-1.12	-1.28
miR-RC-544	1.59	-1.89	2.07	-1.65	-1.30
miR-RC-545	-1.27	-14.68	-1.02	-13.53	-1.28
miR-RC-546	-2.55	-4.41	-2.06	-2.93	-2.53
miR-RC-547	-5.05	-33.69	-1.01	-7.21	-2.55
miR-RC-548	0.00	0.00	0.00	0.00	0.00
miR-RC-549	-2.57	-4.59	-4.11	-2.85	-2.59
miR-RC-550	1.57	-2.03	-1.03	1.01	-1.30
miR-RC-551	1.56	-3.87	1.97	-1.39	-1.26
miR-RC-552	-2.60	-13.35	-8.18	0.00	-10.76
miR-RC-553	1.53	-1.21	-1.05	-1.22	1.53
miR-RC-554	-1.22	-27.94	4.24	-6.43	0.00
miR-RC-555	-1.28	-2.88	-1.01	-3.37	-1.29
miR-RC-556	-3.32	-3.27	-3.36	3.79	2.39
miR-RC-557	1.56	-1.57	-1.00	-1.51	3.18
miR-RC-558	-1.26	-2.01	-2.01	-7.41	-2.79
miR-RC-559	-1.30	-5.61	1.95	-8.63	1.49
miR-RC-560	3.11	1.30	2.08	1.51	3.26

Table 2h) MiRNome expression screening; 1- 104S 1 wk CSFBS, 2- 104S 3 wk CSFBS, 3- 104S 1 wk CDX, 4- 104S 3 wk CDX, 5- 0 hr 104R; All values are comparisons with 0 hr 104S; Green denotes change > 3 fold; Red denotes change < -3 fold

miRNA	1	2	3	4	5
miR-RC-561	-1.37	0.00	2.69	4.49	0.00
miR-RC-562	-2.51	-155.71	-4.05	-6.48	-10.26
miR-RC-563	-1.28	-1.84	-1.04	-1.09	1.54
miR-RC-564	1.57	-6.98	-1.03	-1.61	-5.14
miR-RC-565	-1.24	-2.92	-1.98	-1.97	1.63
miR-RC-566	1.23	1.23	-1.63	-1.03	2.42
miR-RC-567	3.12	1.18	-1.03	1.66	6.22
miR-RC-568	-1.32	-2.52	-1.05	-2.06	-1.30
miR-RC-569	-1.27	-3.60	-2.02	-1.09	-1.28
miR-RC-570	-1.31	-3.86	-2.09	-2.79	-2.63
miR-RC-571	1.59	-1.14	2.01	2.22	1.57
miR-RC-572	-1.27	1.67	-2.14	-1.59	-1.27
miR-RC-573	1.61	1.15	2.08	1.37	1.62
miR-RC-574	2.44	-1.57	2.46	1.83	1.20
miR-RC-575	-1.27	-4.80	3.97	-4.29	1.59
miR-RC-576	1.56	-67.74	-1.01	-1.05	6.35
miR-RC-577	-1.25	-4.77	-4.02	-1.56	-5.71
miR-RC-578	-1.26	-3.44	-4.01	-2.36	-2.51
miR-RC-579	-1.25	-3.14	1.00	-1.74	-2.49
miR-RC-580	1.59	-4.06	1.01	1.06	3.19
miR-RC-581	3.11	-1.60	1.94	1.02	-1.28
miR-RC-582	-1.29	-4.17	-1.01	0.00	-1.26
miR-RC-583	1.57	1.10	-2.03	1.26	3.16
miR-RC-584	-1.28	-3.73	-8.01	-1.98	-1.26
miR-RC-585	-1.30	-1.80	-1.00	1.15	-1.28
miR-RC-586	-1.28	-4.80	-4.44	-2.15	1.53
miR-RC-587	-1.26	-13.36	1.03	-1.61	-1.28
miR-RC-588	-1.25	-6.76	1.83	1.82	1.58
miR-RC-589	-1.32	-3.94	3.93	-6.33	1.52
miR-RC-590	1.60	-3.19	0.00	-25.56	1.58
miR-RC-591	-1.28	-2.44	-2.08	1.48	1.53
miR-RC-592	-1.28	-1.99	-1.02	-2.39	1.54
miR-RC-593	1.61	-2.29	-4.04	-9.82	-1.28
miR-RC-594	3.01	1.94	16.03	4.26	6.39
miR-RC-595	1.57	-1.52	4.13	2.07	1.60
miR-RC-596	-1.65	-1.64	-3.38	-4.73	-3.40
miR-RC-597	-1.63	-1.65	-1.67	1.01	-3.23
miR-RC-598	-5.14	-9.46	1.97	-4.32	-2.54
miR-RC-599	1.58	-2.50	-1.01	-36.59	-1.27
miR-RC-600	-2.52	-1.02	1.00	-2.29	-1.41

miRNA	1	2	3	4	5
miR-RC-601	-1.26	-1.92	-2.10	-1.24	-1.30
miR-RC-602	1.57	-1.15	-1.03	-1.24	-1.34
miR-RC-603	-2.48	-6.71	1.00	-44.49	-1.30
miR-RC-604	-1.22	-7.98	4.29	-1.94	6.76
miR-RC-605	-1.66	-1.63	-1.65	1.94	1.22
miR-RC-606	-1.27	-1.73	-2.03	-1.04	-1.30
miR-RC-607	-1.31	-2.35	-1.03	1.50	1.57
miR-RC-608	1.53	1.88	-2.04	1.07	-1.28
miR-RC-609	-1.67	-3.22	-3.18	-1.98	-3.20
miR-RC-610	0.00	0.00	0.00	0.00	0.00
miR-RC-611	1.21	1.21	-1.64	-1.04	-1.63
miR-RC-612	3.16	-13.00	3.86	-2.30	6.01
miR-RC-613	-1.63	-3.22	-3.32	-2.03	-3.33
miR-RC-614	0.00	0.00	0.00	0.00	0.00
miR-RC-615	-1.33	-1.90	-4.10	-1.34	-1.33
miR-RC-616	0.00	0.00	0.00	0.00	0.00
miR-RC-617	1.55	-1.21	-1.95	-1.80	-1.27
miR-RC-618	-1.25	-1.65	1.72	-2.39	1.31
miR-RC-619	-1.26	-2.77	-1.00	1.25	1.55
miR-RC-620	2.48	-1.60	-1.60	3.89	-3.31
miR-RC-621	1.59	-1.88	1.99	1.14	-1.26
miR-RC-622	-1.24	-10.82	0.00	2.47	1.47
miR-RC-623	-1.24	-8.31	1.85	4.80	3.39
miR-RC-624	-2.53	-28.41	-2.09	-3.77	-1.31
miR-RC-625	1.60	1.35	-2.12	-1.13	1.55
miR-RC-626	1.57	-1.63	-1.93	-1.65	1.56
miR-RC-627	-1.26	-2.35	-1.01	-1.04	-1.29
miR-RC-628	1.61	1.15	1.97	-10.37	-1.28
miR-RC-629	3.09	-7.12	1.95	-1.38	-1.27
miR-RC-630	3.06	-2.92	7.80	3.32	3.14
miR-RC-631	-1.26	0.00	0.00	-1.19	-1.46
miR-RC-632	-1.13	-8.12	1.99	3.65	14.32
miR-RC-633	-4.98	0.00	1.02	-1.58	1.61
miR-RC-634	-1.63	-3.17	-1.62	-1.99	-3.14
miR-RC-635	-1.23	1.23	2.03	1.27	1.59
miR-RC-636	-1.60	-1.65	-1.66	-2.09	-1.65
miR-RC-637	1.60	1.77	-1.12	-1.06	1.56
miR-RC-638	-10.24	-3.78	-1.01	-1.80	-2.53
miR-RC-639	-2.52	-198.47	-2.15	-3.06	1.65
miR-RC-640	1.60	-225.38	-2.02	-1.79	-5.18

Table 2i) MiRNome expression screening; 1- 104S 1 wk CSFBS, 2- 104S 3 wk CSFBS, 3- 104S 1 wk CDX, 4- 104S 3 wk CDX, 5- 0 hr 104R; All values are comparisons with 0 hr 104S; Green denotes change > 3 fold; Red denotes change < -3 fold

miRNA	1	2	3	4	5
miR-RC-641	-1.26	-4.83	-2.04	-2.49	-1.27
miR-RC-642	1.56	1.08	-2.00	1.50	1.54
miR-RC-643	-3.29	-3.20	-1.68	-2.08	-1.66
miR-RC-644	-1.20	-1.81	-1.86	-1.94	-2.39
miR-RC-645	1.53	-1.31	-2.00	1.48	1.51
miR-RC-646	1.63	1.00	-2.02	1.01	1.56
miR-RC-647	-2.65	-3.84	-2.28	-2.07	1.58
miR-RC-648	-1.31	-4.88	-8.52	-2.59	-2.57
miR-RC-649	-3.32	-3.24	-6.57	-143.51	-6.67
miR-RC-650	-5.21	-12.96	-2.14	-2.93	1.54
miR-RC-651	1.57	0.00	1.98	1.50	3.09
miR-RC-652	-1.26	0.00	-1.18	2.50	3.10
miR-RC-653	-1.29	0.00	1.99	-7.13	-1.25
miR-RC-654	1.61	0.00	-1.03	-3.63	1.48
miR-RC-655	0.00	0.00	0.00	0.00	0.00
miR-RC-656	1.56	6.07	-2.04	6.71	3.19
miR-RC-657	-1.22	-2.02	-2.00	-1.92	1.60
miR-RC-658	-2.52	0.00	-2.07	-1.88	1.58
miR-RC-659	1.61	-2.07	-2.00	-1.37	-1.27
miR-RC-660	1.54	-8.51	-1.01	1.57	0.00
miR-RC-661	1.63	-1.07	1.00	1.13	-1.26
miR-RC-662	-2.64	-1.39	-4.74	-1.76	-2.55
miR-RC-663	1.61	-3.12	-3.95	-1.20	1.60
miR-RC-664	1.59	1.91	0.00	1.17	3.15
miR-RC-665	0.00	0.00	0.00	0.00	0.00
miR-RC-666	0.00	0.00	0.00	0.00	0.00
miR-RC-667	0.00	-1.60	1.11	0.00	1.02
miR-RC-668	0.00	2.45	4.86	4.79	7.74
miR-RC-669	0.00	2.52	5.03	2.42	3.96
miR-RC-670	0.00	-3.13	-1.72	0.00	-2.27
miR-RC-671	40.39	1.22	1.25	2.42	-1.03
miR-RC-672	0.00	0.00	0.00	0.00	0.00
miR-RC-673	0.00	0.00	0.00	0.00	0.00
miR-RC-674	-17.98	1.26	-1.64	1.22	-1.02
miR-RC-675	-3.04	-3.25	-1.57	-3.27	-1.01
miR-RC-676	2.35	1.24	5.03	1.23	2.02
miR-RC-677	-1.60	-1.63	1.29	-1.60	-1.01
miR-RC-678	-3.31	-3.18	-13.08	-1.01	-1.65
miR-RC-679	4.72	2.51	5.11	1.27	3.97
miR-RC-680	-6.08	-1.75	1.17	2.63	33.72

miRNA	1	2	3	4	5
miR-RC-681	-1.64	-3.22	-3.28	-2.01	-1.67
miR-RC-682	-16.74	-1.72	1.16	2.47	-1.01
miR-RC-683	0.00	0.00	0.00	0.00	0.00
miR-RC-684	1.01	1.25	-1.65	1.25	-1.03
miR-RC-685	2.37	-1.02	0.00	6.62	10.66
miR-RC-686	0.00	0.00	0.00	0.00	0.00
miR-RC-687	0.00	0.00	0.00	0.00	0.00
miR-RC-688	-1.42	-1.64	-1.61	-1.67	-1.04
miR-RC-689	0.00	0.00	0.00	0.00	0.00
miR-RC-690	0.00	0.00	0.00	0.00	0.00
miR-RC-691	0.00	0.00	0.00	0.00	0.00
miR-RC-692	-1.27	-3.26	0.00	0.00	0.00
miR-RC-693	23.53	6.57	53.17	26.59	5.32
miR-RC-694	-1.05	1.24	-1.62	-1.64	1.92
miR-RC-695	0.00	-1.61	-1.60	-1.68	1.97
miR-RC-696	1.95	1.24	2.52	4.83	7.80
miR-RC-697	0.00	0.00	0.00	0.00	0.00
miR-RC-698	173.48	-1.77	2.33	1.16	1.86
miR-RC-699	-3.47	1.26	-3.20	-3.20	-4.26
miR-RC-700	1.62	1.22	2.54	-6.58	1.93
miR-RC-701	1.62	1.24	4.97	1.21	1.92
miR-RC-702	791.17	-1.75	1.27	1.23	-1.04
miR-RC-703	0.00	-1.60	-1.59	1.22	-1.01
miR-RC-704	-2.32	-1.63	-1.62	-1.65	1.96
miR-RC-705	-1.26	1.23	-13.03	-1.66	1.94
miR-RC-706	0.00	0.00	0.00	0.00	0.00
miR-RC-707	-28.29	-1.61	4.94	2.46	-1.04
miR-RC-708	0.00	0.00	0.00	0.00	0.00
miR-RC-709	0.00	0.00	0.00	0.00	0.00
miR-RC-710	0.00	0.00	0.00	0.00	0.00
miR-RC-711	0.00	0.00	0.00	0.00	0.00
miR-RC-712	-6.27	1.25	-3.36	-3.65	0.00
miR-RC-713	-4.74	-3.33	-1.62	-1.65	1.94
miR-RC-714	-12.89	-3.28	-1.60	-1.64	3.79
miR-RC-715	-1.98	-1.35	1.50	0.00	1.11
miR-RC-716	-3.76	2.50	-12.98	2.44	2.03
miR-RC-717	-77.17	0.00	-1.59	0.00	-4.08
miR-RC-718	0.00	0.00	0.00	0.00	0.00
miR-RC-719	1.44	-1.68	9.72	4.79	7.75
miR-RC-720	0.00	0.00	0.00	0.00	0.00

Table 2j) MiRNome expression screening; 1- 104S 1 wk CSFBS, 2- 104S 3 wk CSFBS, 3- 104S 1 wk CDX, 4- 104S 3 wk CDX, 5- 0 hr 104R; All values are comparisons with 0 hr 104S; Green denotes change > 3 fold; Red denotes change < -3 fold

miRNA	1	2	3	4	5
miR-RC-721	1.09	1.22	1.21	1.18	-1.05
miR-RC-722	-1.66	-3.22	-3.25	-2.01	-3.25
miR-RC-723	0.00	0.00	0.00	0.00	0.00
miR-RC-724	-2.48	-3.22	1.24	1.24	1.96
miR-RC-725	-1.00	-1.65	1.24	1.20	1.94
miR-RC-726	-1.33	1.25	1.26	5.01	1.88
miR-RC-727	2.07	2.49	1.28	2.44	8.03
miR-RC-728	-1.59	-1.60	-1.63	-1.01	-1.64
miR-RC-729	1.34	-1.62	1.25	1.23	-1.02
miR-RC-730	-4.08	-6.50	-6.54	-6.86	-4.07
miR-RC-731	-1.14	1.23	1.17	1.18	1.90
miR-RC-732	-2.90	-1.63	-1.62	-3.31	-36.31
miR-RC-733	0.00	0.00	0.00	0.00	0.00
miR-RC-734	7.06	-1.59	1.27	-1.62	-1.03
miR-RC-735	-1.05	1.22	1.23	-3.32	-2.08
miR-RC-736	-6.39	-1.67	-3.26	-3.35	1.93
miR-RC-737	-1.28	0.00	-3.22	-3.30	-1.02
miR-RC-738	2.72	2.48	2.44	-1.75	3.92
miR-RC-739	1.98	-1.63	1.23	2.42	1.93
miR-RC-740	0.00	0.00	0.00	0.00	0.00
miR-RC-741	0.00	0.00	0.00	0.00	0.00
miR-RC-742	-1.54	-1.68	-1.67	-1.66	-1.05
miR-RC-743	2.42	2.55	1.28	1.23	4.08
miR-RC-744	-1.18	-1.62	-1.61	1.23	1.95
miR-RC-745	1.13	1.31	2.57	1.13	1.98
miR-RC-746	-1.41	-1.64	-3.25	-1.71	-4.20
miR-RC-747	-2.20	-1.59	0.00	-7.22	-2.06
miR-RC-748	-1.31	1.26	-1.60	1.24	-1.03
miR-RC-749	1.53	-1.62	1.24	1.23	2.01
miR-RC-750	4093.22	-1.68	-1.66	-3.17	-1.04
miR-RC-751	-28.54	-1.63	-1.60	2.41	-1.02
miR-RC-752	2.44	4.97	2.41	7.92	1.20
miR-RC-753	-2.11	1.24	1.21	-1.66	-1.03
miR-RC-754	-1.40	-3.26	1.25	-1.61	1.93
miR-RC-755	2.04	2.48	2.54	2.44	1.93
miR-RC-756	0.00	0.00	0.00	0.00	0.00
miR-RC-757	1.15	-3.23	1.24	1.21	-2.06
miR-RC-758	-1.04	-1.65	1.23	2.42	-1.05
miR-RC-759	-1.91	-1.66	-3.28	-31.79	-1.03
miR-RC-760	0.00	0.00	0.00	1.86	0.00

miRNA	1	2	3	4	5
miR-RC-761	4.93	2.49	4.91	7.85	-3.31
miR-RC-762	-27.91	0.00	-6.42	-3.19	-1.03
miR-RC-763	1.19	1.25	-1.68	1.99	2.48
miR-RC-764	-1.68	0.00	4.89	1.09	1.90
miR-RC-765	-1.11	-3.34	1.27	2.37	3.93
miR-RC-766	-1.04	2.50	5.12	4.89	3.91
miR-RC-767	-1.67	2.47	0.00	1.23	4.03
miR-RC-768	-2.90	-1.59	-3.34	-1.74	-2.07
miR-RC-769	1.21	-1.62	-1.64	-4.15	-1.60
miR-RC-770	1.94	1.22	1.23	1.21	3.85
miR-RC-771	-1.28	-1.59	-13.33	-1.66	-1.03
miR-RC-772	1.45	2.56	5.45	1.25	1.97
miR-RC-773	0.00	0.00	0.00	0.00	0.00
miR-RC-774	2.43	1.27	2.48	2.47	1.92
miR-RC-775	1.75	-1.56	2.48	-1.70	-2.19
miR-RC-776	-1.86	-1.59	1.23	1.23	-1.03
miR-RC-777	-1.32	-1.61	1.23	-1.61	3.99
miR-RC-778	0.00	-1.64	-1.63	-1.65	-4.14
miR-RC-779	-4.78	-1.59	-3.14	-1.63	-1.03
miR-RC-780	-1.88	-3.25	-1.63	-1.63	-1.03
miR-RC-781	10.43	5.08	20.43	10.02	7.89
miR-RC-782	4.34	-1.42	1.42	2.75	2.22
miR-RC-783	1.54	1.26	1.24	1.23	2.01
miR-RC-784	3.87	1.34	2.72	1.26	2.08
miR-RC-785	-1.85	-6.50	-1.59	1.20	-2.13
miR-RC-786	-1.18	-1.62	-3.13	-1.59	-1.02
miR-RC-787	1.89	-3.22	1.27	-1.67	-1.06
miR-RC-788	-1.33	1.20	1.20	1.16	-2.15
miR-RC-789	-3.42	0.00	-1.64	1.22	-1.06
miR-RC-790	-1.38	-1.60	-3.29	-1.64	-1.04
miR-RC-791	-1.67	5.14	1.30	1.17	2.01
miR-RC-792	-2.06	-1.62	-1.61	-1.62	-2.08
miR-RC-793	-1.28	0.00	0.00	1.23	0.00
miR-RC-794	-1.94	-3.26	-3.23	1.22	-1.03
miR-RC-795	-1.17	-1.63	1.25	-1.71	1.88
miR-RC-796	0.00	0.00	0.00	0.00	0.00
miR-RC-797	0.00	0.00	0.00	0.00	0.00
miR-RC-798	2.21	2.59	1.28	2.56	2.04
miR-RC-799	1.06	1.23	2.55	2.47	-8.11
miR-RC-800	-1.26	-1.62	1.22	1.22	1.95

Table 2k) MiRNome expression screening; 1- 104S 1 wk CSFBS, 2- 104S 3 wk CSFBS, 3- 104S 1 wk CDX, 4- 104S 3 wk CDX, 5- 0 hr 104R; All values are comparisons with 0 hr 104S; Green denotes change > 3 fold; Red denotes change < -3 fold

miRNA	1	2	3	4	5
miR-RC-801	1.14	2.52	2.54	4.96	3.89
miR-RC-802	-5.44	-6.56	-3.26	-3.24	-4.13
miR-RC-803	-2.78	-1.62	1.22	-1.69	-1.06
miR-RC-804	-1.85	-1.57	-1.62	-3.31	-2.10
miR-RC-805	-2.08	-1.56	-3.25	-3.21	-2.00
miR-RC-806	-1.65	5.04	2.48	4.90	1.98
miR-RC-807	4.84	10.07	4.90	15.46	4.88
miR-RC-808	-3.71	-1.61	-1.59	-3.33	-1.05
miR-RC-809	-1.21	1.25	1.22	1.24	2.01
miR-RC-810	-1.75	-3.31	-1.64	-1.65	-2.11
miR-RC-811	-1.25	-1.59	-1.64	-1.54	-1.06
miR-RC-812	-2.58	-1.62	-1.61	-1.61	-2.11
miR-RC-813	1.22	1.22	1.26	1.92	1.23
miR-RC-814	-2.50	-11.93	-1.97	-8.26	1.60
miR-RC-815	1.43	-3.22	1.27	1.21	15.57
miR-RC-816	2.49	1.20	1.24	7.93	2.57
miR-RC-817	1.23	-1.63	-1.65	-1.01	1.21
miR-RC-818	0.00	0.00	0.00	0.00	0.00
miR-RC-819	-5.54	-1.65	-1.66	-1.69	-2.06
miR-RC-820	-2.24	-1.62	-3.22	-1.63	-1.05
miR-RC-821	-1.62	-1.58	-3.19	-1.95	-3.17
miR-RC-822	1.26	2.53	2.50	-1.73	-1.05
miR-RC-823	-1.23	-1.58	1.27	-1.62	-1.02
miR-RC-824	-14.03	-6.56	-6.69	-3.38	-9.57
miR-RC-825	-14.87	-13.04	-1.66	-1.64	-1.03
miR-RC-826	-1.18	1.25	-1.05	2.55	1.96
miR-RC-827	-3.20	1.22	-1.67	1.21	1.91
miR-RC-828	1.39	1.21	1.20	0.00	-1.03
miR-RC-829	-1.36	1.25	-1.60	-1.62	-1.03
miR-RC-830	-1.82	1.23	-1.60	1.21	-2.08
miR-RC-831	0.00	0.00	0.00	0.00	0.00
miR-RC-832	1.85	-1.58	2.53	1.23	2.06
miR-RC-833	-8.78	-3.22	-1.63	-3.31	-2.04
miR-RC-834	-2.06	-3.24	1.23	-1.63	-2.06
miR-RC-835	-1.23	-1.59	-1.66	0.00	0.00
miR-RC-836	0.00	0.00	0.00	0.00	0.00
miR-RC-837	4.37	4.97	10.23	4.97	3.91
miR-RC-838	-1.22	1.25	-1.60	-1.63	-2.04
miR-RC-839	1.24	-1.62	1.24	-1.03	-1.69
miR-RC-840	-2.51	-3.21	1.23	-1.65	-4.08

miRNA	1	2	3	4	5
miR-RC-841	-2.82	-6.52	-3.34	-3.34	-2.07
miR-RC-842	1.53	-3.28	1.25	-3.19	-2.03
miR-RC-843	-2.01	-3.17	1.27	1.23	-16.53
miR-RC-844	-1.65	1.21	-1.61	-1.65	-1.03
miR-RC-845	-1.13	0.00	2.44	1.21	7.79
miR-RC-846	-1.59	1.23	-3.51	1.98	-1.64
miR-RC-847	-4.36	0.00	0.00	0.00	-1.01
miR-RC-848	-1.25	-1.59	1.25	1.23	-1.04
miR-RC-849	-1.18	-1.67	1.23	1.22	-1.08
miR-RC-850	0.00	0.00	0.00	0.00	0.00
miR-RC-851	0.00	0.00	0.00	0.00	0.00
miR-RC-852	-1.01	1.37	5.30	1.13	1.07
miR-RC-853	-2.57	-19.77	-2.00	-9.88	-1.29
miR-RC-854	1.25	1.25	1.21	1.95	2.42
miR-RC-855	1.72	1.21	2.46	2.32	-1.04
miR-RC-856	-1.01	1.21	1.24	1.21	1.95
miR-RC-857	1.03	-4.02	-1.62	2.45	1.97
miR-RC-858	1.21	-1.61	-1.60	1.22	-1.02
miR-RC-859	-1.29	-7.27	-1.99	-6.14	1.59
miR-RC-860	2.43	2.44	1.24	3.93	1.19
miR-RC-861	5.35	2.66	0.00	17.40	10.78
miR-RC-862	-1.23	-8.42	-1.95	-7.84	-2.56
miR-RC-863	2.37	1.21	2.45	1.96	1.17
miR-RC-864	-1.27	-4.24	-1.03	-2.73	1.55
miR-RC-865	1.25	1.25	1.25	1.95	1.22
miR-RC-866	-15.39	-1.41	-23.30	1.30	1.14
miR-RC-867	-1.03	1.27	1.25	1.25	-1.98
miR-RC-868	-1.07	1.23	1.24	-3.24	-1.05
miR-RC-869	-1.43	-1.61	-3.25	1.21	1.01
miR-RC-870	-1.92	-3.18	-3.22	-3.23	-2.03
miR-RC-871	-2.05	-1.62	-3.23	-1.64	-1.04
miR-RC-872	-1.74	-1.62	-1.60	-1.62	-1.01
miR-RC-873	-2.27	1.26	-1.56	-1.58	-2.04
miR-RC-874	-107.21	0.00	-2.91	-3.15	-1.57
miR-RC-875	-4.44	-3.29	-3.24	-3.39	-2.12
miR-RC-876	-2.63	-3.21	0.00	-1.63	-2.07
miR-RC-877	-17.26	0.00	0.00	0.00	-1.03
miR-RC-878	-2.61	-2.67	-1.02	-19.98	3.10
miR-RC-879	3.17	-1.59	2.07	-2.02	3.10
miR-RC-880	5.17	2.48	2.48	4.05	1.25

Table 2l) MiRNome expression screening; 1- 104S 1 wk CSFBS, 2- 104S 3 wk CSFBS, 3- 104S 1 wk CDX, 4- 104S 3 wk CDX, 5- 0 hr 104R; All values are comparisons with 0 hr 104S; Green denotes change > 3 fold; Red denotes change < -3 fold

miRNA	1	2	3	4	5
miR-RC-881	6.25	-17.64	4.01	-1.40	3.13
miR-RC-882	1.55	-1.89	-1.02	1.07	-2.54
miR-RC-883	1.20	-1.63	1.21	1.89	1.18
miR-RC-884	1.58	-3.21	1.03	-1.83	-2.59
miR-RC-885	-3.27	-3.20	-3.30	-1.03	-1.60

Complete linkage hierarchical clustering analysis was carried out using miRNAs that had fold amplification data for at least three of the five groups in Table 2. The horizontal order of miRNAs and the vertical order of treatment groups were arranged into clusters, with more similar elements ordered closer together in the heat map, presented in Figure 2. Up-regulated expression appears as red and down-regulated expression appears as green. Hierarchical cluster dendrograms were constructed for the miRNAs and treatments to visualize how individual miRNAs and treatments were grouped. It can be seen that distinct clusters of up- and down-regulated miRNA genes emerged from the hierarchical clustering exercise. Of the miRNAs included the in analysis, 199 exhibited general patterns of up-regulation with treatment, and 599 exhibited general patterns of down-regulation. A cluster of 99 miRNAs showed a consistent pattern of strong up-regulation and a cluster of 241 miRNAs showed a consistent pattern of strong down-regulation.

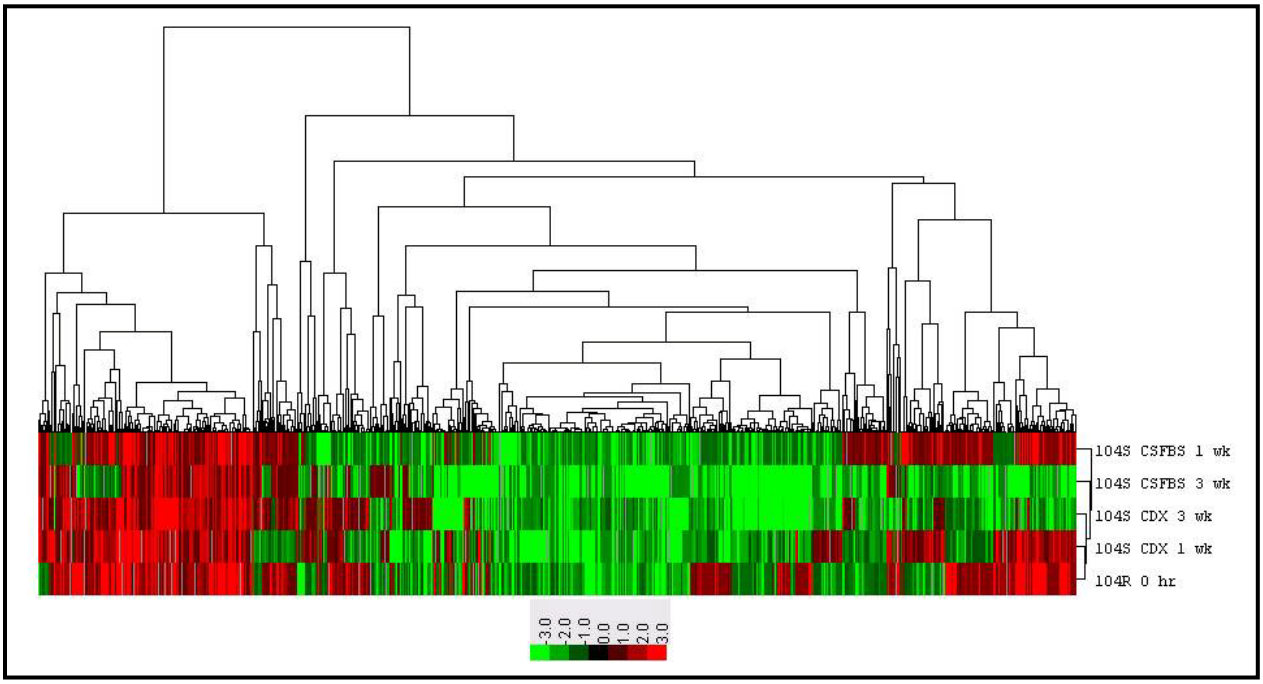

Figure 2) Heat map of clustered miRNA expression across all treatment groups using LNCaP zero hour expression as reference

The raw Ct data was then processed by the ΔΔCt method using different treatment groups as reference groups than had been previously been used in an attempt to better analyze miRNA changes that occur due to the effects of CDX-treatment. Fold amplification values were determined according to the comparisons listed in Table1. The miRNAs were ranked according to fold amplification values for each comparison. Tables 3 and 4 contain the top ranked up-regulated and down-regulated results, respectively. The miRNAs that are included in these tables have fold-amplification values that are at least two standard deviations removed from the mean change in expression for the comparison (this corresponds to z-scores ≥ 2.0 or ≤ -2.0; refer to Materials and Methods for a description of the method for determining z-scores). MiRNAs that have fold amplification values greater than three standard deviations above the mean are denoted with green backgrounds in Table 3, and those with fold amplification values greater than three standard deviations below the mean are denoted with red backgrounds in Table 3. The letter denotation of each comparison can be referenced in Table 1. Assigning significance based on z-score proved to be a more conservative approach than the three-fold method and decreased the total number of miRNAs considered significant; the category containing the greatest number of significant miRNAs was the down-regulated portion of comparison D with 56 miRNAs, and the up-regulated portion of comparison D included only 8 miRNAs. The average fold-changes in expression for comparisons A, B, and C were slightly negative (μ_A=0.959, μ_C=0.778, μ_D=0.608), but none exceeded more than 1.7-fold down-regulation. Comparison A had an average fold-change in expression of 1.43.

Table 3) Up-regulated miRNAs in comparisons A through D with expression fold changes greater than two standard deviations above the mean fold change; μ is the geometric mean of all amplified miRNAs in the comparison; σ is one geometric standard deviation

A (μ=0.959; σ=2.663)			B (μ=1.433; σ=2.257)			B (cont.)			C (μ=0.778; σ=3.801)			D (μ=0.608; σ=4.328)		
miRNA	Fold Δ	z-score	miRNA	Fold Δ	z-score	miRNA	Fold Δ	z-score	miRNA	Fold Δ	z-score	miRNA	Fold Δ	z-score
miR-RC-707	139.904	52.17	miR-RC-640	126.045	55.22	miR-RC-719	8.028	2.92	miR-RC-716	31.687	8.15	miR-RC-517	96.241	22.15
miR-RC-797	107.412	39.97	miR-RC-639	64.772	28.07	miR-RC-46	8.003	2.91	miR-RC-866	30.232	7.77	miR-RC-309	75.127	17.28
miR-RC-717	48.520	17.86	miR-RC-576	64.595	27.99	miR-RC-825	7.943	2.88	miR-RC-7	27.613	7.08	miR-RC-197	45.283	10.38
miR-RC-104	46.810	17.22	miR-RC-175	43.463	18.63	miR-RC-765	7.926	2.88	miR-RC-656	13.664	3.41	miR-RC-761	26.014	5.93
miR-RC-796	45.321	16.66	miR-RC-623	39.884	17.04	miR-RC-785	7.788	2.82	miR-RC-238	13.454	3.35	miR-RC-843	20.278	4.60
miR-RC-244	38.361	14.05	miR-RC-375	31.999	13.55	miR-RC-624	7.541	2.71	miR-RC-28	13.323	3.32	miR-RC-799	20.060	4.55
miR-RC-874	36.830	13.47	miR-RC-272	31.257	13.22	miR-RC-308	7.103	2.51	miR-RC-678	12.985	3.23	miR-RC-620	12.876	2.89
miR-RC-682	19.424	6.93	miR-RC-632	29.618	12.49	miR-RC-685	6.741	2.35	miR-RC-392	12.976	3.23	miR-RC-732	10.982	2.46
miR-RC-751	17.855	6.35	miR-RC-622	26.715	11.20	miR-RC-161	6.618	2.30	miR-RC-283	12.884	3.20			
miR-RC-672	17.732	6.30	miR-RC-141	26.223	10.99	miR-RC-816	6.591	2.29	miR-RC-556	12.752	3.17			
miR-RC-39	16.622	5.88	miR-RC-562	24.037	10.02	miR-RC-453	6.571	2.28						
miR-RC-674	10.951	3.75	miR-RC-477	16.259	6.57	miR-RC-861	6.541	2.26						
miR-RC-487	10.240	3.49	miR-RC-66	15.454	6.21	miR-RC-101	6.482	2.24						
miR-RC-638	10.143	3.45	miR-RC-660	13.348	5.28	miR-RC-283	6.466	2.23						
miR-RC-598	10.143	3.45	miR-RC-881	12.634	4.96	miR-RC-345	6.446	2.22						
miR-RC-412	9.605	3.25	miR-RC-556	12.400	4.86	miR-RC-485	6.353	2.18						
miR-RC-825	8.970	3.01	miR-RC-588	12.322	4.83	miR-RC-414	6.343	2.18						
miR-RC-764	8.233	2.73	miR-RC-523	12.212	4.78	miR-RC-620	6.219	2.12						
miR-RC-686	8.150	2.70	miR-RC-57	12.021	4.69	miR-RC-420	6.016	2.03						
miR-RC-135	8.063	2.67	miR-RC-857	9.833	3.72	miR-RC-211	5.985	2.02						
miR-RC-714	8.051	2.66	miR-RC-630	9.679	3.65									
miR-RC-680	7.120	2.31	miR-RC-26	9.218	3.45									
miR-RC-719	6.734	2.17	miR-RC-22	9.101	3.40									
			miR-RC-587	8.293	3.04									

Table 4) Down-regulated miRNAs in comparisons A through D with negative expression fold changes greater than two standard deviations below the mean fold change; μ is the geometric mean of all amplified miRNAs in the comparison; σ is one geometric standard deviation

A (μ=0.959; σ=2.663)			B (μ=1.433; σ=2.257)			C (μ=0.778; σ=3.801)			D (μ=0.608; σ=4.328)			D (cont.)		
miRNA	Fold Δ	z-score	miRNA	Fold Δ	z-score	miRNA	Fold Δ	z-score	miRNA	Fold Δ	z-score	miRNA	Fold Δ	z-score
miR-RC-750	-6803.123	-2554.19	miR-RC-301	-54.745	-24.01	miR-RC-529	-238.747	-62.48	miR-RC-343	-247.413	-56.78	miR-RC-559	-12.843	-2.59
miR-RC-702	-625.426	-234.46	miR-RC-649	-44.227	-19.35	miR-RC-343	-160.910	-42.00	miR-RC-407	-246.141	-56.49	miR-RC-815	-12.812	-2.58
miR-RC-698	-74.536	-27.60	miR-RC-291	-31.656	-13.78	miR-RC-301	-155.977	-40.70	miR-RC-220	-196.495	-45.02	miR-RC-680	-12.799	-2.58
miR-RC-671	-32.382	-11.77	miR-RC-442	-24.971	-10.81	miR-RC-300	-83.018	-21.50	miR-RC-258	-101.411	-23.05	miR-RC-700	-12.707	-2.56
miR-RC-199	-12.938	-4.47	miR-RC-759	-19.182	-8.25	miR-RC-247	-82.761	-21.44	miR-RC-344	-98.509	-22.38	miR-RC-491	-12.396	-2.48
miR-RC-538	-12.720	-4.38	miR-RC-599	-14.615	-6.23	miR-RC-528	-79.186	-20.50	miR-RC-529	-93.553	-21.23	miR-RC-399	-12.333	-2.47
miR-RC-771	-10.398	-3.51	miR-RC-628	-11.978	-5.06	miR-RC-407	-78.055	-20.20	miR-RC-452	-79.758	-18.05	miR-RC-216	-11.553	-2.29
miR-RC-705	-10.349	-3.49	miR-RC-220	-8.132	-3.35	miR-RC-442	-77.699	-20.11	miR-RC-329	-75.883	-17.15	miR-RC-349	-11.434	-2.26
miR-RC-7	-8.456	-2.78	miR-RC-590	-8.016	-3.30	miR-RC-12	-66.693	-17.21	miR-RC-256	-65.804	-14.82	miR-RC-185	-10.593	-2.07
miR-RC-392	-7.998	-2.61	miR-RC-700	-8.016	-3.30	miR-RC-220	-60.427	-15.56	miR-RC-301	-62.032	-13.95	miR-RC-545	-10.529	-2.05
miR-RC-514	-7.970	-2.60	miR-RC-878	-7.480	-3.06	miR-RC-452	-51.386	-13.18	miR-RC-878	-61.915	-13.92	miR-RC-379	-10.484	-2.04
miR-RC-283	-7.949	-2.59	miR-RC-407	-6.739	-2.74	miR-RC-537	-51.034	-13.09	miR-RC-442	-60.019	-13.49	miR-RC-36	-10.402	-2.02
miR-RC-593	-6.524	-2.06	miR-RC-603	-6.628	-2.69	miR-RC-603	-44.602	-11.40	miR-RC-12	-52.320	-11.71	miR-RC-387	-10.369	-2.02
miR-RC-648	-6.489	-2.05	miR-RC-528	-5.396	-2.14	miR-RC-599	-36.110	-9.16	miR-RC-291	-49.230	-10.99			
miR-RC-12	-6.413	-2.02				miR-RC-258	-32.857	-8.31	miR-RC-590	-40.408	-8.96			
miR-RC-375	-6.383	-2.01				miR-RC-291	-31.520	-7.96	miR-RC-75	-39.833	-8.82			
						miR-RC-303	-28.897	-7.26	miR-RC-603	-34.296	-7.54			
						miR-RC-554	-27.297	-6.84	miR-RC-528	-32.675	-7.17			
						miR-RC-589	-24.882	-6.21	miR-RC-448	-31.625	-6.93			
						miR-RC-326	-23.954	-5.96	miR-RC-759	-30.923	-6.76			
						miR-RC-329	-23.456	-5.83	miR-RC-599	-28.751	-6.26			
						miR-RC-649	-21.837	-5.41	miR-RC-78	-25.914	-5.61			
						miR-RC-628	-20.458	-5.04	miR-RC-534	-24.335	-5.24			
						miR-RC-22	-19.763	-4.86	miR-RC-303	-23.472	-5.04			
						miR-RC-878	-19.571	-4.81	miR-RC-450	-23.140	-4.97			
						miR-RC-527	-18.482	-4.52	miR-RC-327	-22.925	-4.92			
						miR-RC-575	-17.001	-4.13	miR-RC-178	-21.722	-4.64			
						miR-RC-559	-16.799	-4.08	miR-RC-649	-21.529	-4.59			
						miR-RC-700	-16.718	-4.06	miR-RC-227	-20.340	-4.32			
						miR-RC-653	-14.161	-3.39	miR-RC-328	-19.817	-4.20			
						miR-RC-545	-13.323	-3.17	miR-RC-422	-17.159	-3.58			
						miR-RC-207	-10.587	-2.45	miR-RC-375	-16.784	-3.50			
						miR-RC-256	-10.457	-2.41	miR-RC-304	-16.399	-3.41			
						miR-RC-465	-10.434	-2.41	miR-RC-465	-16.243	-3.37			
						miR-RC-209	-9.984	-2.29	miR-RC-288	-15.419	-3.18			
						miR-RC-497	-9.875	-2.26	miR-RC-527	-14.528	-2.98			
						miR-RC-328	-9.753	-2.23	miR-RC-612	-13.803	-2.81			
						miR-RC-170	-9.692	-2.21	miR-RC-306	-13.783	-2.80			
						miR-RC-759	-9.678	-2.21	miR-RC-29	-13.483	-2.74			
						miR-RC-495	-9.267	-2.10	miR-RC-231	-13.352	-2.70			
						miR-RC-286	-9.185	-2.08	miR-RC-814	-13.199	-2.67			
						miR-RC-396	-9.010	-2.03	miR-RC-604	-13.097	-2.65			
									miR-RC-173	-13.071	-2.64			

Significantly deregulated miRNAs were then analyzed in a manner illustrated by the Venn diagrams in Figure 3. All miRNAs that showed three-fold deregulation in any of the comparison groups of Table 1 were included in the Venn diagrams for either up- or down-regulated miRNAs, and a small number of miRNAs were included in both diagrams. The numbers in the figure represent the number of miRNAs in a specific section of the Venn diagram. In all, 60 miRNAs were up-regulated in Comparison B (three weeks CDX vs. three weeks CS-FBS) and at least one other comparison group. 17 miRNAs were up-regulated in CDX treated cells compared to CS-FBS-only treated cells at both one week and three weeks timepoints, Comparisons A and B. Two miRNAs, miR-RC-751 and miR-RC-308, also showed significant up-regulation between the one week and three weeks CDX timepoints, Comparison C. Several miRNAs included in the Venn diagram analysis had also been deemed significant due to z-scores greater than 2.0 or less than -2.0, and these miRNAs are identified in red in Figure 3 and Figure 4.

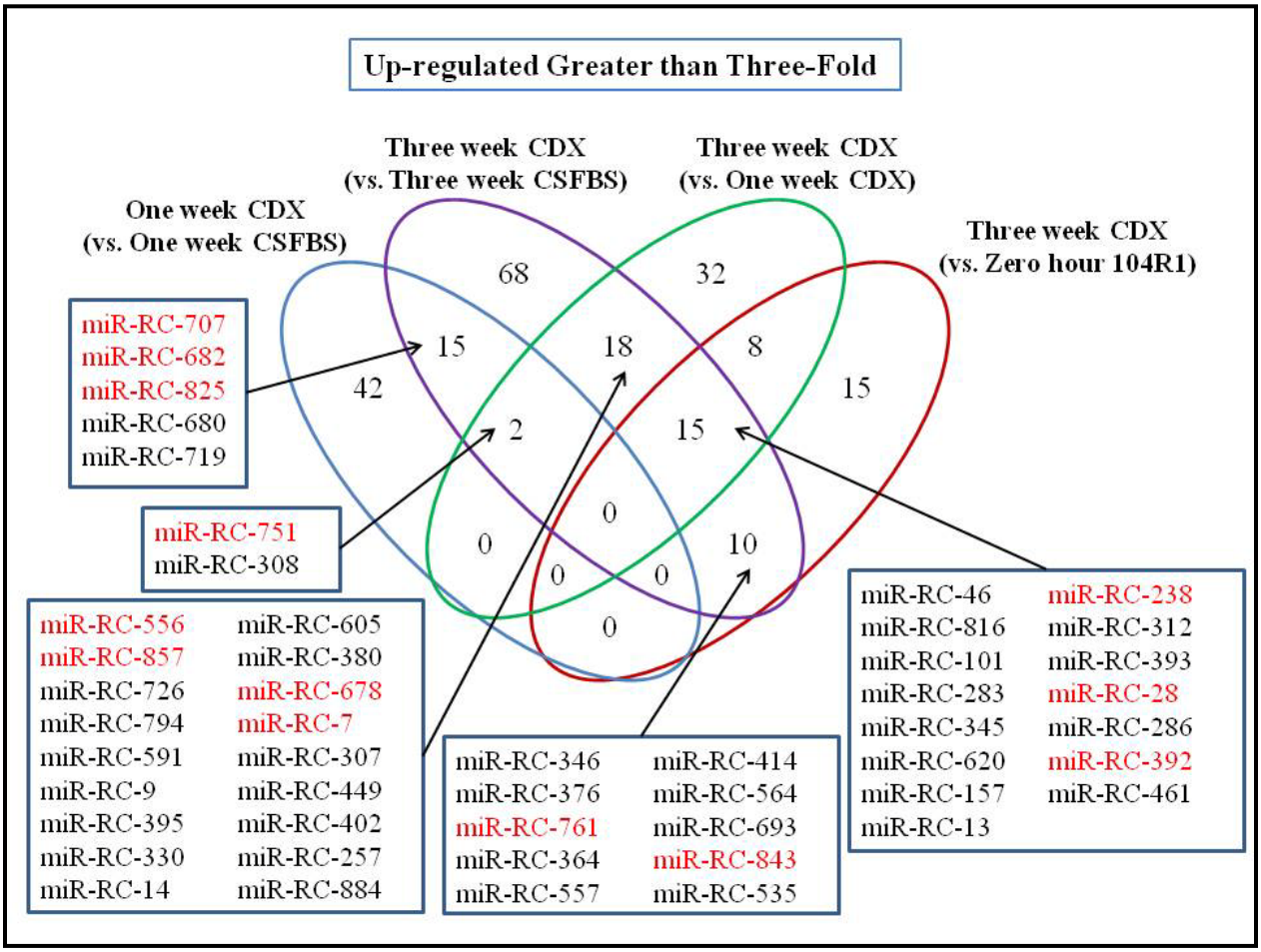

Figure 3) Venn diagram cross-comparison analysis of up-regulated miRNAs for comparisons A through D; miRNAs in red have fold changes of two standard deviations or greater above the mean fold change in at least one comparison

The down-regulated miRNAs in Figure 4 consists of 29 miRNAs that exhibited three-fold or greater down-regulation in Comparison B and either or both Comparison C and/or Comparison D. Only one miRNA, miR-RC-593, was significantly down-regulated at both the one week and three weeks CDX timepoints, Comparisons A and B. MiR-RC-593 was also down-regulated in Comparison D, CDX treatment compared to androgen insensitive cells.

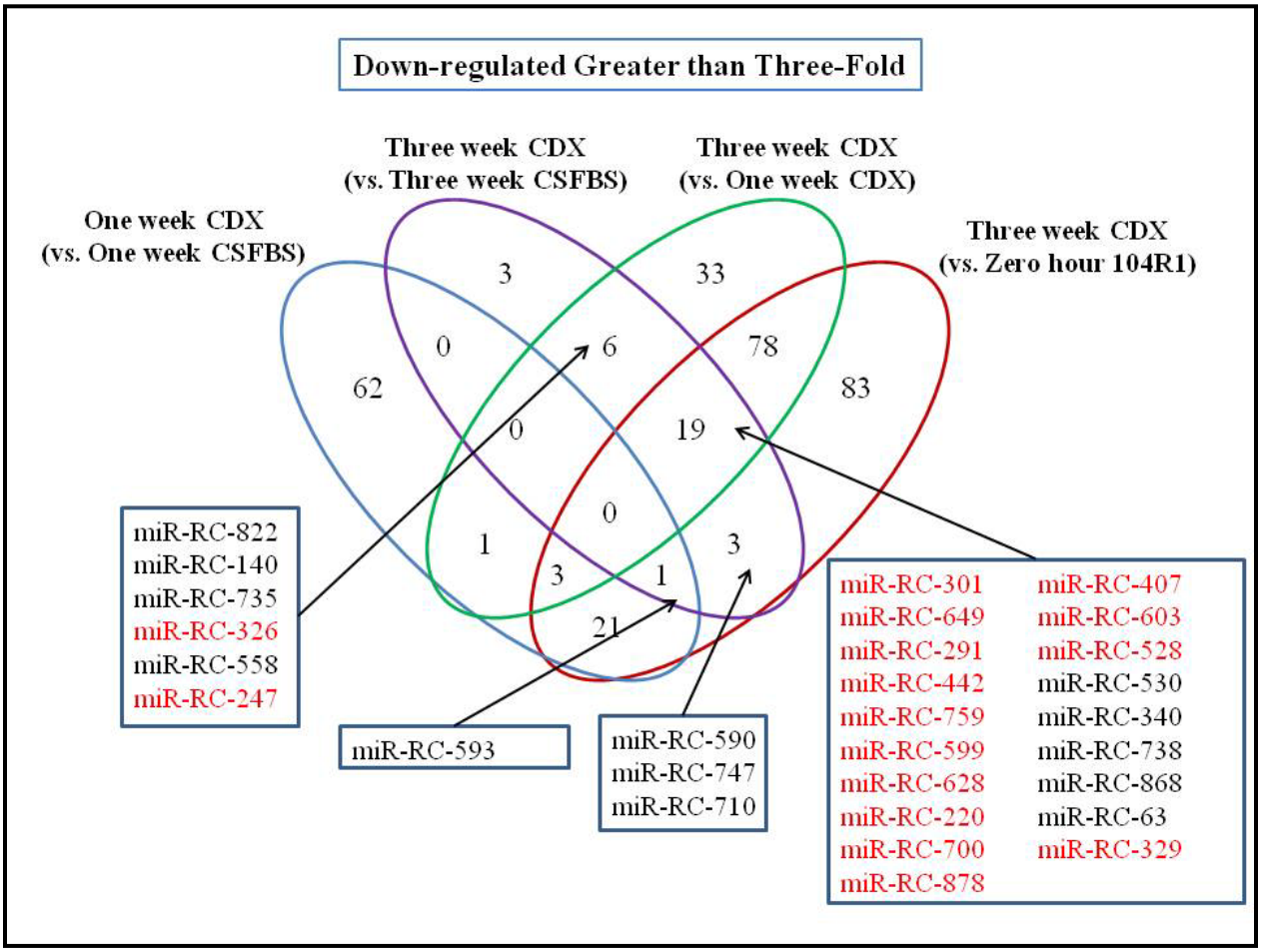

Figure 4) Venn diagram cross-comparison analysis of down-regulated miRNAs for comparisons A through D; miRNAs in red have fold changes of two standard deviations or greater below the mean fold change in at least one comparison

The primary objective of this study was to identify a group of miRNAs which show significantly altered expression during CDX treatment to be used for developing clinical screening procedures as well as for further investigation of cancer progression mechanisms. These candidate miRNAs were identified based on the previously described comparison methods and are presented in Table 5 as an array of primary candidate miRNAs. Up-regulated primary candidate miRNAs were chosen from the top-ranked miRNAs that had expression values greater than two standard deviations above the mean expression change in CDX treated cells at three weeks, compared to CS-FBS treated cells or to zero hour LNCaP-104R1 cells, Comparisons B and D. Since none of the very highly ranked up-regulated miRNAs were up-regulated in multiple comparisons, none of the miRNAs appearing in Figure 3 were included in the array of primary candidates. Candidate miRNAs for the down-regulated group were chosen on the condition that they showed at least two standard deviations of down-regulation in any comparison and also at least three-fold down-regulation in CDX treated cells at three weeks. Again, the top ranked miRNAs were given preference. We chose 28 primary candidate miRNAs, with 14 exhibiting overall up-regulation and 14 exhibiting overall down-regulation. The number of candidate miRNAs included in the primary candidates array was intentionally kept relatively low to facilitate results in the next stage of data analysis, target prediction. The order of miRNAs presented in Table 5 cannot objectively be considered the order of significance.

Table 5) Primary candidate array of most significantly deregulated candidate miRNAs

Primary Candidate MicroRNAs	
Up-regulated	**Down-regulated**
miR-RC-640	miR-RC-301
miR-RC-639	miR-RC-649
miR-RC-576	miR-RC-291
miR-RC-175	miR-RC-442
miR-RC-623	miR-RC-759
miR-RC-375	miR-RC-599
miR-RC-272	miR-RC-220
miR-RC-632	miR-RC-878
miR-RC-622	miR-RC-407
miR-RC-141	miR-RC-603
miR-RC-562	miR-RC-528
miR-RC-517	miR-RC-329
miR-RC-309	miR-RC-490
miR-RC-197	miR-RC-247

To serve as a more complete collection of candidate miRNAs for future screening purposes, a second array of miRNAs was constructed, referred to as the secondary array of candidate miRNAs and presented in Table 6. This array encompassed all the miRNAs included in the primary array, plus other significantly deregulated candidates. Any miRNA that had z-scores greater 3.0 or less than -3.0 and any miRNA that was at least three-fold deregulated in Comparison B and one other comparison (all miRNAs listed in Figure 3 and Figure 4) were included as elements in the secondary array of candidate miRNAs. The secondary array consists of 102 upregulated miRNAs and 85 down-regulated miRNAs.

Table 6) Secondary candidate array of significantly deregulated candidate miRNAs

Secondary Candidate MicroRNAs						
Up-regulated				Down-regulated		
miR-RC-707	miR-RC-376	miR-RC-104	miR-RC-881	miR-RC-822	miR-RC-750	miR-RC-258
miR-RC-682	miR-RC-761	miR-RC-796	miR-RC-556	miR-RC-140	miR-RC-702	miR-RC-303
miR-RC-825	miR-RC-364	miR-RC-244	miR-RC-588	miR-RC-735	miR-RC-698	miR-RC-554
miR-RC-680	miR-RC-557	miR-RC-874	miR-RC-523	miR-RC-326	miR-RC-671	miR-RC-589
miR-RC-719	miR-RC-414	miR-RC-751	miR-RC-57	miR-RC-558	miR-RC-199	miR-RC-326
miR-RC-751	miR-RC-564	miR-RC-672	miR-RC-857	miR-RC-247	miR-RC-538	miR-RC-329
miR-RC-308	miR-RC-693	miR-RC-39	miR-RC-630	miR-RC-593	miR-RC-771	miR-RC-22
miR-RC-556	miR-RC-843	miR-RC-674	miR-RC-26	miR-RC-590	miR-RC-301	miR-RC-527
miR-RC-857	miR-RC-535	miR-RC-487	miR-RC-22	miR-RC-747	miR-RC-649	miR-RC-575
miR-RC-726	miR-RC-46	miR-RC-638	miR-RC-587	miR-RC-710	miR-RC-291	miR-RC-559
miR-RC-794	miR-RC-816	miR-RC-598	miR-RC-716	miR-RC-301	miR-RC-442	miR-RC-653
miR-RC-591	miR-RC-101	miR-RC-412	miR-RC-866	miR-RC-649	miR-RC-759	miR-RC-545
miR-RC-9	miR-RC-283	miR-RC-640	miR-RC-656	miR-RC-291	miR-RC-599	miR-RC-344
miR-RC-395	miR-RC-345	miR-RC-639	miR-RC-238	miR-RC-442	miR-RC-628	miR-RC-256
miR-RC-330	miR-RC-620	miR-RC-576	miR-RC-28	miR-RC-759	miR-RC-220	miR-RC-75
miR-RC-14	miR-RC-157	miR-RC-175	miR-RC-678	miR-RC-599	miR-RC-590	miR-RC-448
miR-RC-605	miR-RC-13	miR-RC-623	miR-RC-392	miR-RC-628	miR-RC-700	miR-RC-78
miR-RC-380	miR-RC-238	miR-RC-375	miR-RC-283	miR-RC-220	miR-RC-878	miR-RC-534
miR-RC-678	miR-RC-312	miR-RC-272	miR-RC-517	miR-RC-700	miR-RC-529	miR-RC-450
miR-RC-7	miR-RC-393	miR-RC-632	miR-RC-309	miR-RC-878	miR-RC-343	miR-RC-327
miR-RC-307	miR-RC-28	miR-RC-622	miR-RC-197	miR-RC-407	miR-RC-300	miR-RC-178
miR-RC-449	miR-RC-286	miR-RC-141	miR-RC-761	miR-RC-603	miR-RC-247	miR-RC-227
miR-RC-402	miR-RC-392	miR-RC-562	miR-RC-843	miR-RC-528	miR-RC-528	miR-RC-328
miR-RC-257	miR-RC-461	miR-RC-477	miR-RC-799	miR-RC-530	miR-RC-407	miR-RC-422
miR-RC-884	miR-RC-797	miR-RC-66		miR-RC-340	miR-RC-12	miR-RC-375
miR-RC-346	miR-RC-717	miR-RC-660		miR-RC-738	miR-RC-452	miR-RC-304
				miR-RC-868	miR-RC-537	miR-RC-465
				miR-RC-63	miR-RC-603	miR-RC-288
				miR-RC-329		

The top miRDB (miRNA DataBase, http://mirdb.org) target prediction results for the miRNAs of the up-regulated and down-regulated lists of the primary array of candidates are presented in Table 7 and Table 8, respectively. The miRDB score is the cumulative target score for a single miRNA and a single mRNA transcript assigned by the internet-based prediction software miRDataBase, with 100 being the highest score possible. The TargetScan Human column is checked if the miRNA-target prediction is validated by internet-based prediction database TargetScan Human. Refer to Materials and Methods for a description of how targets were chosen for inclusion in Figure 7 and Figure 8. Several genes are predicted targets of more than three up-regulated primary candidate miRNAs, including SH3TC2, CLASP2, AAK1, GTF2H1, and NARG1. The genes ZEB1 and ZEB2 are targeted by miR-RC-517 with a miRDB score of 100, the highest score possible. Also of note, the genes ZEB1, ZEB2, SLC7A11, TET2, TMEM170B, UBE2B, ALS2CR2, CYP20A1, ERRFI1, SH3TC2, SLC35B4, TNRC6B, ZKSCAN1, and ZNF826 are targeted by at least one up-regulated miRNA with a score of 99.0 or greater.

Table 7) Predicted target genes of up-regulated miRNAs; TS column shows validation of target match with the TargetScan Human database (miR-1914 is not included in the TargetScan Human database)

Target Gene	miRNA Name	miRDB Score	TS
ZEB1	miR-RC-517	100.0	√
	miR-RC-309	99.0	
ZEB2	miR-RC-517	100.0	√
	miR-RC-309	94.0	√
VASH2	miR-RC-517	99.0	√
SLC7A11	miR-RC-375	99.0	√
	miR-RC-517	76.0	√
TET2	miR-RC-375	99.0	√
	miR-RC-623	67.0	
TMEM170B	miR-RC-309	99.0	√
	miR-RC-517	75.0	√
UBE2B	miR-RC-623	99.0	√
	miR-RC-517	83.0	√
ALS2CR2	miR-RC-375	99.0	√
CYP20A1	miR-RC-623	99.0	
ERRFI1	miR-RC-517	99.0	√
SH3TC2	miR-RC-623	99.0	
	miR-RC-640	91.0	√
	miR-RC-622	89.0	√
	miR-RC-175	78.0	√
	miR-RC-141	76.0	√
	miR-RC-309	60.0	√
SLC35B4	miR-RC-517	99.0	√
TNRC6B	miR-RC-375	99.0	√
	miR-RC-309	96.0	√
	miR-RC-632	70.0	√
ZKSCAN1	miR-RC-623	99.0	√
ZNF826	miR-RC-197	99.0	√
CLASP2	miR-RC-375	86.0	√
	miR-RC-197	80.0	√
	miR-RC-309	84.0	√
	miR-RC-517	90.0	√
AAK1	miR-RC-175	83.0	√
	miR-RC-623	99.0	√
	miR-RC-562	73.0	√
	miR-RC-309	79.0	√
GTF2H1	miR-RC-640	81.0	√
	miR-RC-272	73.0	
	miR-RC-576	98.0	√
	miR-RC-622	75.0	√
NARG1	miR-RC-640	71.0	√
	miR-RC-375	95.0	√
	miR-RC-562	66.0	√
	miR-RC-622	65.0	√
ELAVL2	miR-RC-623	87.0	√
	miR-RC-517	89.0	√
	miR-RC-309	90.0	√
C6orf120	miR-RC-640	83.0	√
	miR-RC-622	83.0	√
	miR-RC-517	93.0	√
LHFP	miR-RC-141	72.0	√
	miR-RC-517	96.0	√
	miR-RC-309	88.0	√
YWHAG	miR-RC-562	65.0	√
	miR-RC-309	90.0	√
	miR-RC-517	97.0	√
PALM2	miR-RC-623	72.0	
	miR-RC-562	93.0	√
	miR-RC-517	84.0	√
HIPK2	miR-RC-640	92.0	√
	miR-RC-175	65.0	√
	miR-RC-622	91.0	√
GALNT14	miR-RC-175	68.0	√
	miR-RC-640	90.0	√
	miR-RC-622	89.0	√
HS2ST1	miR-RC-623	69.0	
	miR-RC-141	85.0	√
	miR-RC-309	93.0	√
IPO5	miR-RC-640	75.0	√
	miR-RC-622	75.0	√
	miR-RC-309	94.0	√
RAB22A	miR-RC-576	68.0	√
	miR-RC-623	98.0	√
	miR-RC-562	78.0	√
ABL2	miR-RC-375	82.0	√
	miR-RC-562	66.0	
	miR-RC-309	95.0	√

Down-regulated miRNAs of the primary candidate array have 11 very significant predicted targets, but only ZNF776 is targeted by more than two miRNAs and receives at least one score of 80.0 or above. The genes LATS2 and ZKSCAN1 are both predicted targets of miR-RC-407 and have miRDB scores of 99.0. The genes ZKSCAN1 and SH3TC2 are strongly predicted targets of miRNAs belonging to both the up-regulated and down-regulated lists of the primary candidates array.

Table 8) Predicted target genes of down-regulated miRNAs; TS column shows validation of target match with the TargetScan Human database (miR-1972 is not included in the TargetScan Human database)

Target Gene	miRNA Name	miRDB Score	TS
LATS2	miR-RC-407	99.0	√
ZKSCAN1	miR-RC-407	99.0	√
LPAR4	miR-RC-590	98.0	√
	miR-RC-220	67.0	√
UBE2B	miR-RC-407	98.0	√
VPS53	miR-RC-220	98.0	
ZNF776	miR-RC-301	87.0	√
	miR-RC-220	60.0	√
	miR-RC-407	60.0	√
SH3TC2	miR-RC-590	96.0	√
	miR-RC-407	95.0	√
C1orf21	miR-RC-301	95.0	√
	miR-RC-291	84.0	
GPBP1L1	miR-RC-759	87.0	√
	miR-RC-442	80.0	√
ZBTB41	miR-RC-407	93.0	√
	miR-RC-220	68.0	√
FLJ45831	miR-RC-759	87.0	√
	miR-RC-442	73.0	√

DISCUSSION

In this study we sought to investigate the deregulation of miRNAs in human prostate cancer treated with the anti-androgen drug Casodex and to shed light on the possible functional roles of deregulated miRNAs in the progression of prostate cancer cells toward anti-androgen resistance. Extensive analysis of expression for almost all known human miRNA sequences was carried out, and an array of deregulated miRNAs was identified, as were several putative regulatory target genes. The data suggest that some form of miRNA regulation is at play in the progression of androgen sensitive LNCaP cells to a castration resistant phenotype. Quantitative PCR is the most sensitive method of measuring gene expression available, so large changes measured in the expression of miRNA genes can be taken as a reflection of cellular regulation involving cancer progression processes and the effects of CDX treatment. The comparisons of expression values that were carried out revealed that several miRNAs were up- and down-regulated. Interestingly, the initial ΔΔCt method analysis and cluster analysis revealed that approximately 75% of the miRNAs screened shown some pattern down-regulation when prostate cancer cells were subjected to stimuli of androgen independence. This overall decrease in miRNA production might be a signal of general deregulation of cellular processes, contributing to more aggressive cancer phenotypes.

The miRNAs miR-RC-301, miR-RC-442, miR-RC-291, and miR-RC-599 had the most widespread down-regulation of the miRNAs screened. They were significantly down-regulated in androgen sensitive cells treated with CDX for three weeks compared to androgen sensitive

cells treated only with CS-FBS, and they were also found to be down-regulated in androgen sensitive cells treated with CDX for three weeks compared to androgen insensitive LNCaP-104R1 cells. MiR-RC-599 showed significant decrease in expression between the one week and three week timepoints of CDX treatment in androgen sensitive cells. These expression patterns strongly indicate that these miRNAs have high potential for being implicated in the regulation of progression to the androgen independent phenotype. There are known cancer roles for the miRNAs corresponding to our sequences miR-RC-301 and miR-RC-442. Hsa-miR-199a-5p has been shown to be down-regulated in the progression process of oral cancers in hamster models (Yu *et al.*, 2009). This same down-regulated expression pattern was seen in our cancer progression model. Investigation into therapeutic targets for leukemia has revealed that miR-337 down-regulation is associated with overexpression of the tyrosine kinase Lyn, possibly contributing to the rise of malignant phenotypes in certain types of leukemia (Hussein *et al.*, 2009). Again, this same expression pattern was seen in our model, suggesting that the activity of Lyn kinase could contribute to CDX-resistance.

Another miRNA found to be consistently down-regulated was miR-RC-220. It was recently shown that repression of malignant characteristics and some cell cycle arrest at the G1/S transition are seen when knocked-down expression of this miRNA is restored in hepatocellular carcinoma cells. The same study presented evidence that the miRNA targets the mRNA of CCND2 (Cyclin D2) (Wang *et al.*, 2010). We did not strongly predict CCND2 as a target of miRNA regulation most likely due to the array-based approach taken to target prediction, with preference given to targets that received multiple hits. The down-regulation of miR-RC-220 in

cells progressing to CDX-resistance may lead to decreased inhibition of cyclin D2 function and increased survival due to accelerated G1/S transition.

We also found that miR-RC-590 was down-regulated during progression to CDX-resistance. This result has been corroborated by a study of miRNA expression in clear renal cell carcinoma tissue samples, where it was found that miR-514 (corresponding to our miR-RC-590 sequence) is very highly down-regulated compared to matched non-malignant samples. It is, however, unclear whether low miR-514 levels are part of a cancer affecter mechanism or just the result of an altered regulatory mechanism, as no correlation between miR-514 expression and tumor stage or survival were found (Jung *et al.*, 2009). It was also determined in our study that the miRNA miR-RC-247 (corresponding to hsa-miR-184) is significantly down-regulated during CDX-treatment of androgen sensitive cells. There is evidence that miR-184 has tumor suppressor functions through indirect inhibition of the Akt pathway. It was found that miR-184 can bind to and repress the function of another miRNA, miR-205, which itself represses the enzyme SHIP2 (thought to inhibit Akt signaling) (Yu *et al.*, 2008).

The miRNAs in the up-regulated list of primary candidates demonstrated less consistency in expression across our four comparison analyses, but still resulted in some important findings. Firstly, the two most highly up-regulated miRNAs in androgen sensitive cells treated with CDX for three weeks compared to androgen sensitive cells treated only with CSFBS were miR-RC-640 and miR-RC-639, with 126.045 and 64.772 fold up-regulation respectively. Interestingly, these miRNA sequences arise from the same primary miRNA hairpin, being cleaved from

opposite arms of the hairpin by dicer. Increased expression of the primary hairpin transcript and subsequent retention of both possible strands suggests that these miRNAs are involved in CDX-induced drug resistance. Another miRNA found to be significantly up-regulated in our study was miR-RC-197. This finding is contrary to the pattern found in one study that reported down-regulation of miR-146a (corresponding to miR-RC-197) in androgen independent prostate cancer, leading to increased expression of its regulatory target ROCK1 (Lin *et al.*, 2008). However, it may be the case that miR-146a down-regulation is an effect of depleted androgen levels and that the effects of anti-androgen binding include increased miR-146a expression.

Two members of the miR-200 family exhibited significant differential expression and were included in the primary candidate array of up-regulated miRNAs, miR-RC-517 and miR-RC-309. It has been shown by several studies that up-regulation of these miRNAs are important for migration of certain cancers. Mesenchymal (metastatic) ovarian cancer cells overexpress the miR-200 family miRNA miR-429 compared to non-metastatic ovarian cancer cells. Additionally, the transcription factors ZEB1 and ZEB2 are known targets of miR-429. ZEB1 and ZEB2 levels are decreased in ovarian cancer cells when miR-429 is overexpressed (Chen *et al.*, 2011), corroborating the miRNA expression and target prediction results of our study. MiR-200a has also been implicated in migration through targeting of ZEB2 in nasopharyngeal carcinoma cells (Xia *et al.*, 2010).

The genes LATS2, ZKSCAN1, LPAR4, and VPS53 were the most highly predicted to be regulatory targets of down-regulated miRNAs, suggesting an increase in expression of the genes

in question. LATS2 is a known tumor suppressor gene that has lowered expression in a diverse array of cancers, including prostate cancer. In a genomic study of malignant mesothelioma cell lines, the majority of the studied lines showed deletion or mutation of the LATS2 gene. IT was also found that LATS2 inhibits the YAP oncogene by phosphorylation (Murakami *et al.*, 2011). Another study showed that in breast and prostate tissues decreased expression of LATS2 is possibly due to a defective mutation in the transcription factor FOXP3 (Li *et al.*, 2011). Given the very high score assigned to the miR-RC-407/LATS2 interaction by miRDB (99.0), it is extremely probable that the target prediction is accurate. However, more complex regulatory pathways than can be elucidated by this screening study alone may be at play. Two gene targets were predicted for both up- and down-regulated miRNAs of the primary candidate array. ZKSCAN1 is a strongly predicted target of miR-RC-623 and miR-RC-407. SH3TC2 is a strongly predicted target of multiple up- and down-regulated miRNAs. It is expected that in the case of multiple regulatory miRNAs specific for the same mRNA transcript, the effect of the up-regulated miRNA would be phenotypically apparent, i.e. decreased expression of the target gene.

It is important that the miRNA screening be replicated for the candidate miRNAs chosen from this preliminary investigation in matched samples of patient tumors before and following the acquisition of an androgen independent phenotype. Additionally, the genes predicted as targets in this study should be the focus of further investigations to verify both that expression of these genes are altered upon treatment with CDX and that these genes are indeed targeted by the deregulated miRNAs as predicted.

In conclusion, the genome-wide miRNA expression screening resulted in an array of both up-regulated and down-regulated candidate miRNAs that have altered expression in androgen sensitive LNCaP cells as a result of treatment with CDX, separate from the effects of androgen depletion alone. 28 miRNAs exhibiting differential expression in the process of acquiring drug resistance were compiled into an array of primary miRNA candidates for diagnostic and therapeutic use. These miRNAs could have clinical implications as part of a screening test to more accurately identify the level of progression to drug resistance or to predict the effectiveness of anti-androgen therapy in a patient. Uses of miRNA markers that would lead to more selective use of anti-androgen therapy would certainly be worthwhile if it would make it possible to avoid the certain decline in quality of life for patients undergoing cancer therapy. This study forms the groundwork for further studies to validate the expression profiles of the identified miRNAs and to characterize the putative miRNA-target interactions.

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